ANELLOVIRUSES IN HUMAN AND NON-HUMAN PRIMATES

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To Mum, Dad and Kirsteen.

ABSTRACT

The Anelloviruses Torque Teno virus (TTV) and TTV-like minivirus (TLMV) are small, circular DNA viruses which infect humans and non-human primates. They are highly prevalent in the general population, however infection is without any apparent pathology. Both viruses are extremely heterogeneous, especially for DNA viruses, and the role of the immune system in controlling the infection has yet to be established.

Initial experiments involved establishing prevalence figures for TTV and TLMV as well as SENV D and H, subtypes of TTV implicated in potential transfusion transmitted non A-E hepatitis, in Scottish blood donors. TTV and TLMV were co-amplified by PCR using degenerate primers and the PCR products differentiated by melting curve analysis, a method developed in the course of this work. 88% of serum samples were PCR positive for either TTV, TLMV or a heterogeneous mixture of both viruses. The presence of SENV D and H was determined by Southern blot and revealed 0.5% of samples tested were infected with SENV D, 10.9% with SENV H and 1% with both SENV D and SENV H.

The immunosuppression associated with HIV infection and AIDS leads to an increase in TTV/TLMV titres is serum however, there is no data on the effect of immunosuppression on TTV/TLMV titres in tissues. We compared the titre of both TTV and TLMV in the bone marrow and spleen from 3 groups: HIV negative individuals, HIV positive individuals and HIV positive individuals who had progressed to AIDS, leading to immunosuppression. Limiting dilution PCR using primers situated in the UTR region of the genome were used to semi-quantitate the virus, and TTV and TLMV were differentiated. The AIDS group had higher titres, which were statistically significant compared with both the HIV positive and negative groups for both bone marrow and spleen. Analysis of TTV/TLMV titre with CD4 T lymphocyte count showed a significant inverse correlation. On the other hand, neither HCV co-infection nor type of Anellovirus infection (single TTV or TLMV, or mixed TTV/TLMV) showed any significant correlation with virus titre.

TTV/TLMV titres in HIV positive and transplant patients were compared to individuals infected with viruses not known to cause immunosuppression (HCV and HBV) and healthy blood donors. Both of the immunosuppressed groups of individuals had titres of TTV/TLMV in serum higher than the other three groups.

The suggestion that farm animals were infected with TTV similar to human TTV led to an investigation of TTV/TLMV homologues infecting non-human primates and farm animals. Although a high proportion of the non-human primates were infected with TTV/TLMV-like viruses, none of the farm animals were shown to be PCR positive. Sequence analysis of the primate samples determined they were infected with viruses which were genetically distinct from human TTV and TLMV.

In summary, this investigation has 1) shown a high prevalence of TTV/TLMV in Scottish blood donors and determined SENV D and H prevalence in the same population: 2) established for the first time that the immunosuppression associated with AIDS results in an increased TTV/TLMV titre in bone marrow and spleen: 3) determined Anellovirus titre is higher in the sera of immunosuppressed HIV positive individuals and transplant recipients than in the HCV or HBV positive sera or in healthy individuals and 4) confirmed that non-human primates are infected with species specific TTV/TLMV-like viruses, phylogenetically distinct from human viruses.

DECLARATION

Except where specific reference is made to other sources, the work contained in this thesis is the original work of the author. It has not been submitted in whole, or in part, for any other degree.

Katrina S. Thom

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Finally, special thanks go to Tim who has put up with and encouraged me, and has always been there with a bottle of rum to numb the pain.

ABBREVIATIONS

5'NCR	5' non-coding region
A	adenine
aa	amino acid
ALT	alanine aminotransferase
APS	ammonium persulphate
ATP	adenosine triphosphate
BFDV	Beak and feather disease virus
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CAV	Chicken anemia virus
cDNA	complementary DNA
CMV	Cytomegalovirus
CNS	central nervous system
CPE	cytopathic effect
CSF	cerebrospinal fluid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddNTP	didioxynucleoside triphosphate
dGTP	deoxguanosine triphosphate
dH_20	distilled water
DNA	deoxyribonucleic acid
DR	direct repeat
ds	double stranded
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetraacetic acid
EBV	Epstein-Barr virus
ELISA	enzyme linked immunosorbent assay
EtBr	ethidium bromide
FRET	fluorescence resonance energy transfer
G	guanine

gp	glycoprotein
HCl	hydrochloric acid
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	hepatocellular carcinoma
Hg	mercury
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	Herpes simplex virus
HVR	hypervariable region
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IPTG	isopropyly-thiogalactosidase
IVDU	intravenous drug user
kb	kilobases
KCl	potassium chloride
LB	Luria-Bertani
LED	light emitting diode
mM	millimolar
MS	multiple sclerosis
$MgCl_2$	magnesium chloride
NaCl	sodium chloride
NK	natural killer
nt	nucleotide
OD	optical density
OI	opportunistic infection
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	Porcine circovirus
p.i.	post inoculation

pmol	picomoles
RDA	representational difference analysis
RNA	ribonucleic acid
rpm	revolutions per minute
rt	room temperature
SDS	sodium dodecyl sulphate
SIV	Simian immunodeficiency virus
SLE	systemic lupus erythematosus
SNBTS	Scottish National Blood Transfusion Service
T	thymine
TAIP	TTV-derived apoptosis-inducing protein
Taq	*Thermus aquaticus*
TBE	tris-borate EDTA
TEMED	N,N,N',N,-tetramethyethyediamine
Tfl	*Thermus flavus*
TLMV	TTV-like minivirus
Tm	melting temperature
TTV	Torque teno virus
UTR	untranslated region
UV	ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

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Chapter 1

Introduction

1 INTRODUCTION

New human viruses are periodically described and examined for potential causal links with conditions of unknown etiology. In the course of these investigations, the original aims may not be fully satisfied however, unique virological properties may be discovered which advance the field of virology.

Torque teno virus (TTV) and TTV-like minivirus (TLMV) are small circular DNA viruses that infect humans, non-human primates and some domesticated animals. Humans can be simultaneously infected with multiple genotypes of TTV and TLMV and remain viraemic for extended periods of time. They display an unusual degree of heterogeneity for DNA viruses, which has made investigation of any genotype specific disease association problematic.

1.1 Discovery and characterisation

1.1.1 TT Virus

TTV was first isolated from the serum of a Japanese patient (initials T. T.) who developed non A-G hepatitis following a blood transfusion administered during cardiac surgery. Representational difference analysis was used to compare a serum sample taken two weeks post transfusion when the patient had normal alanine aminotransferase (ALT) levels with a sample taken between seven and nine weeks later when the patient's ALT levels had become elevated. This resulted in the discovery of a 500bp clone (N22 clone) that contained one putative open reading frame (ORF) encoding 166 amino acids (aa), which showed no significant homology to any published sequence. The sequence was used to generate primers for polymerase chain reaction (PCR) and these were used to confirm the novel sequence was not genomic in origin.

Ultracentrifugation in a sucrose density gradient showed the N22 sequence corresponded to nucleic acids which appeared in fractions with a peak at 1.26 g/cm^3, indicating that it was associated with a viral particle. The density of the particle was unchanged following treatment with Tween demonstrating the virus (TTV) was not enveloped [Nishizawa et al., 1997]. In order to determine if the TTV genome was composed of RNA or DNA, nucleic acids were extracted from TTV positive plasma and digested with a variety of nucleases. TTV was sensitive to DNase I and resistant to RNase A suggesting that TTV was a DNA virus. This was further substantiated by the sensitivity of TTV to mung bean nuclease,

which has an affinity for single stranded DNA [Okamoto, 1998]. The polarity of the genome was confirmed as negative sense using a hybridisation/nuclease protection assay and the viral particle diameter estimated to be between 30 and 50nm as determined by PCR amplification of infected serum after it was passed through polycarbonate filters with decreasing pore sizes [Mushahwar et al., 1999]. Virus-like particles with a diameter of between 30 and 32nm have also been visualised from TTV infected serum using electron microscopy [Itoh et al., 2000]. Cloning and sequencing of the DNA initially suggested the virus was linear with a genome of 3739 bases but further investigation found an additional 113 nucleotide GC rich region which acts as a bridge to join either end of the genome forming a covalently closed circular structure [Miyata et al., 1999]. This is the first single stranded, circular DNA virus known to infect humans.

1.1.2 TTV-like minivirus

TTV-like minivirus was discovered by serendipity in 1999, two years after TTV was first identified, when PCR primers designed to amplify TTV annealed to, and amplified, a homologous region of TLMV. The plasma tested was co-infected with both viruses and it was only due to a discrepancy with the PCR conditions that TLMV was preferentially amplified over TTV. TLMV can be amplified without reverse transcription and was sensitive to mung bean nuclease suggesting that this too was a single stranded DNA virus. Analysis of the serum on a caesium chloride density gradient showed the viruses could be found in separate fractions, indicating that TLMV genome was not incorporated into the TTV virion. TLMV is non-enveloped and the genome is circular and of negative polarity but, at less than 30nm, it is smaller in diameter than TTV. When sequenced, the full-length genome was 2860 nucleotides in length, approximately one kb smaller than TTV [Takahashi et al., 2000b].

1.1.3 SEN virus

SEN virus, like TTV, was originally identified as a potential viral cause for hepatitis of unknown etiology and was found during a mass screen of some 600 stored blood samples. It was first detected in, and named after, an Italian intra-venous drug user (IVDU) who was infected with Human immunodeficiency virus-1 (HIV). Preliminary data showed a significantly increased proportion of individuals with non A-E hepatitis harbouring the virus compared with the group of healthy blood donors tested simultaneously, but subsequent studies showed that like TTV and TLMV, SEN-V can be found at relatively

high frequencies in healthy individuals. Although initially thought to be a member of a novel group of viruses, closer investigation showed SENV to be a small, unenveloped, single stranded DNA virus and genomic analysis revealed it was a subtype of TTV [Tanaka et al., 2001].

1.2 Classification

It has been problematic to classify TTV and TLMV into a suitable virus family. Traditionally single stranded DNA viruses with no lipid envelopes that infect vertebrates were classified as either *Parvoviridae,* a group of linear DNA viruses, which include the human virus parvovirus B19, or *Circoviridae,* which are circular DNA viruses. Upon further investigation, the similarities of TTV to CAV (family *Circoviridae*, genus Gyrovirus) became more apparent, as shown in Table 1.2.1 [Hijikata et al., 1999]. The subsequent discovery of a 113bp GC rich segment, which forms a linker to make the TTV and TLMV genome circular, excluded them from the Parvovirus family.

Property	TTV	TLMV	CAV	B19
Virion features	Unenveloped, icosahedral	Unenveloped, icosahedral	Unenveloped, icosahedral	Unenveloped, icosahedral
Virion size	30-32nm	29-30nm	23-25nm	18-22nm
Genome type	Single stranded DNA	Single stranded DNA	Single stranded DNA	Single stranded DNA
Genome size	~3.8kb	~2.9kb	~2.3kb	~5kb
Genome form	Covalently closed, circle	Covalently closed, circle	Covalently closed, circle	Linear, with inverted repeats at both ends
Genome polarity	Negative	Negative	Negative	Negative and positive
Predicted open reading frames	ORF1, ORF2, (ORF3?), ORF4 & ORF5	ORF1, ORF2, ORF4 & ORF5	ORF1, ORF2, ORF3 & ORF4	ORF1, ORF2 & ORF3

Table 1.2.1 – Comparison of TTV and TLMV with representative members of *Parvoviridae* (B19) and *Circoviridae* (CAV).

The family *Circoviridae* contains a number of small, single stranded, circular DNA viruses which infect domesticated animal and bird species. This family includes Porcine circovirus (PCV) and Beak and feather disease virus (BFDV) both of which have a diameter of 15 to 20nm and genomes of 2kb. Chicken anemia virus (CAV), which has a slightly larger capsid of 23 to 25nm and genome of 2.3kb, is also a member of this family and it is this virus in particular which shares characteristics with TTV and TLMV. TTV, TLMV and CAV show similarities in the genome organisation, particularly in the spatial arrangements of the open reading frames ORF1 and ORF2 which are partially overlapping in all three viruses.

In 2002 the International Committee for the Taxonomy of Viruses proposed that TTV and TLMV should form the founder members of a new genus Anellovirus, derived from 'anello' meaning 'ring' relating to the circular nature of the genome. Although the similarity to CAV was acknowledged, it was decided that TTV and TLMV were not significantly similar to members of *Circoviridae* other than CAV to warrant the inclusion of these viruses in that family.

They also proposed a new name for TTV: Torque teno virus from 'torques', the necklace, and 'tenuis', thin, which relates to the single stranded, circular nature of the genome. Also suggested was the change from TTV-like mini virus to Torque teno mini virus (TTMV). For the purpose of this thesis TTV-like mini virus (TLMV) will continue to be used.

1.3 Genome organisation and mRNAs

1.3.1 TTV/SENV

On the discovery of TTV, the lack of any single stranded circular viruses infecting humans resulted in the comparison of TTV with members of *Circoviridae*. The Circovirus CAV has a larger capsid and genome than other members of this family, and appears to share a similar genomic organisation with both TTV and TLMV (Figure 1.3.1) including a partially overlapping ORF1 and ORF2. TTV has a negative sense single stranded DNA genome of approximately 3750 – 3900 nucleotides which may contain up to 5 open reading frames and a ~1.2kb untranslated region. The non-coding region of the genome contains a 113 nucleotide GC rich stretch of DNA which includes transcriptional regulatory motifs [Kamada et al., 2004; Miyata et al., 1999].

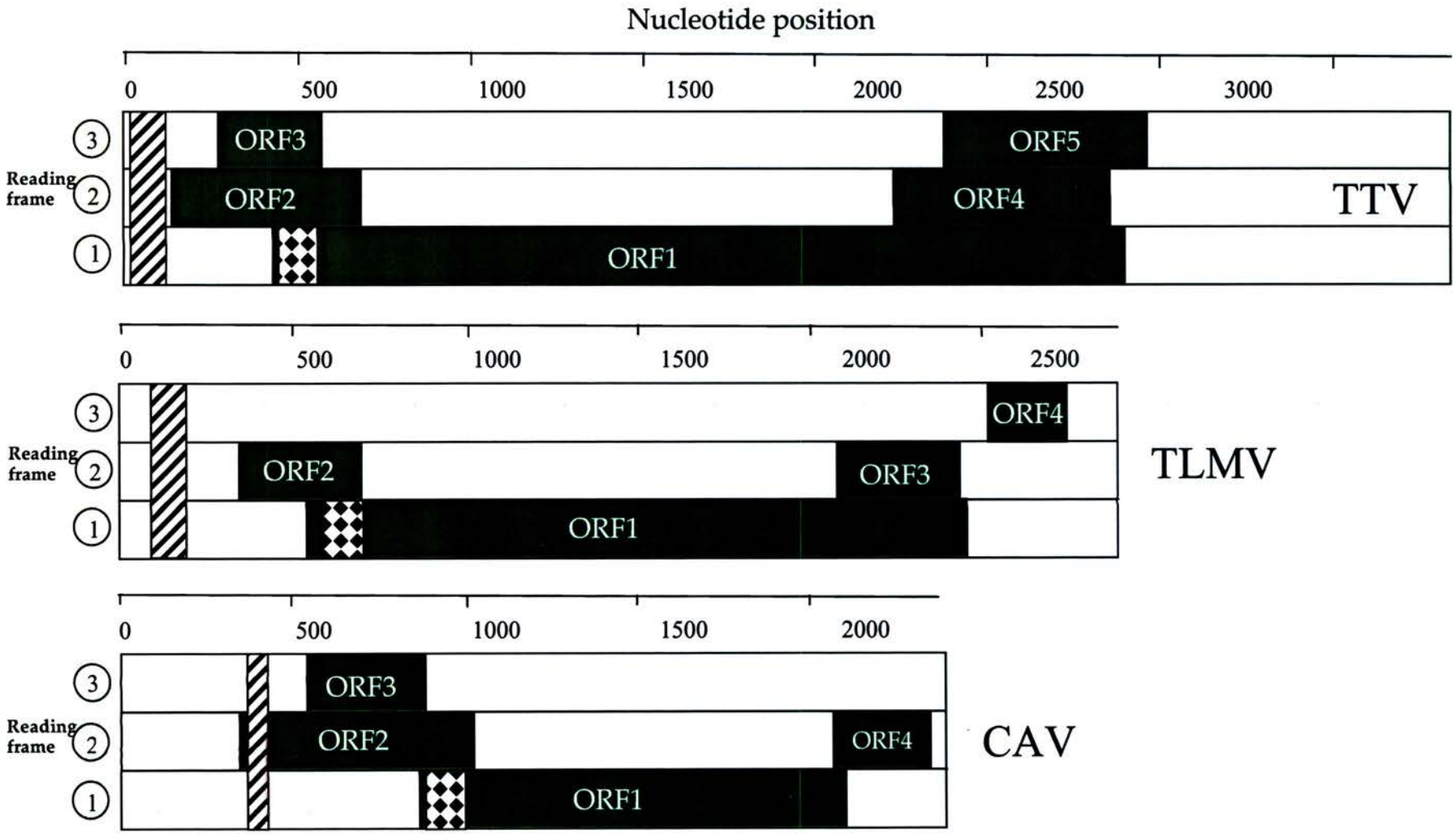

Figure 1.3.1 – Schematic diagram of genome organisation of TTV, TLMV and CAV. Coding regions are shown in green and non- coding regions in white. All three share partially overlapping reading frames ORF1 and ORF2. Diagonal stripes show position of partially conserved UTR and diamonds indicate an arginine rich region of amino acids.

This portion of the genome also contains the only nucleotide sequence which has a high degree of homology with CAV, a 36 nucleotide region which shares 80.6% homology [Takahashi, 1998].

Until recently, the lack of an appropriate culture system for TTV [Desai, 2005] has hampered the analysis of the transcription profile, however transfection of a COS cell line with a plasmid containing permuted whole genome constructs of TTV produced three species of mRNAs. Northern blotting detected mRNAs of 2.8, 1.2 and 1.0 kb 48 hours post transfection. All three mRNAs share common 3' and 5' termini (Figure 1.3.2) and are thought to be transcribed from the same internal promoter, TATA box and cap site [Kamahora et al., 2000; Okamoto et al., 2000c]. Investigation of the TYM9 strain (accession number AB050448) found a common splicing pattern, which joined nucleotides 181 to 283, occurred in all three mRNA species. Transcription initiation for the major protein coding sequence in frame 1, ORF1, is thought to occur at the first ATG at position 559 therefore is not affected by this common splice site. This produces a 748 amino acid product. ORF2, on the other hand, is likely to start at the third ATG in that frame, codon 338, as the first two initiation codons are located in the spliced region spanning nucleotides 181 to 283. ORF2, which is in reading frame two, encodes a 115 amino acid protein. The 1.2 and 1.0 kb mRNAs contained a second splice site which joins nucleotide 681 to nucleotides 2341 and 2579 respectively. This results in the in-frame joining of ORF2 and ORF4, producing a product of 260 amino acids. The linking of ORF2 and ORF5 results in a frame shift and a 249 amino acid product [Kamahora et al., 2000].

This pattern of splicing seen *in vitro* was corroborated by a study which used reverse transcription to detect all three species of mRNA *in vivo* from bone marrow cells infected with TTV [Okamoto et al., 2000c]. The genotype of TTV found in the bone marrow cells was considerably different to the prototype TTV strain, sharing only 67% nucleotide homology, however the shared transcription profile suggests that despite the heterogeneity of the virus the splice sites remain common to all genotypes.

More recently, transfection of a full length TTV clone has shown that the TTV genome can generate at least six proteins by translating each of the three species of mRNA from both of the AUG start codons (at nucleotides 581 and 354 for genotype 6) [Qiu et al., 2005].

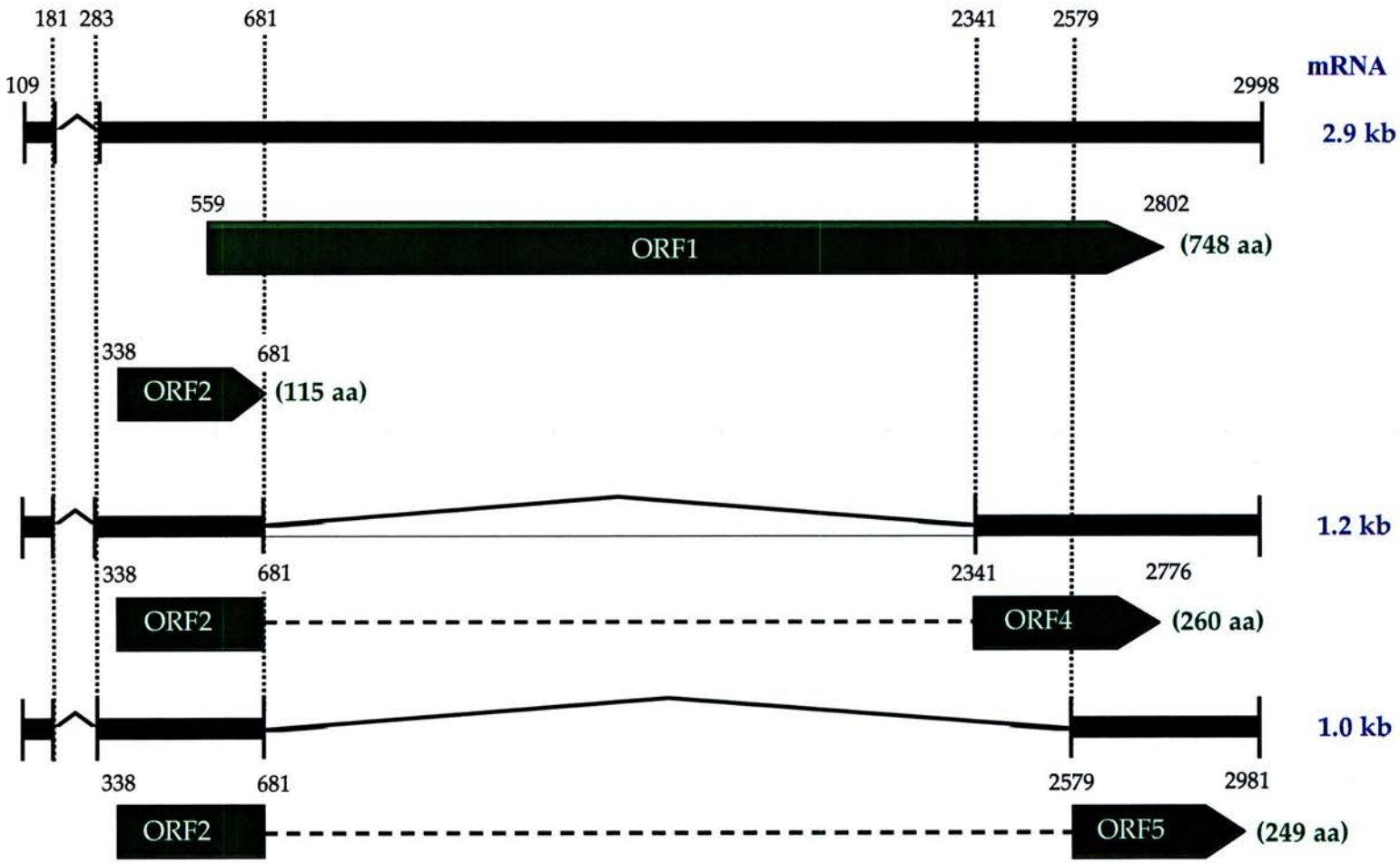

Figure 1.3.2 – TTV mRNA species configurations and predicted open reading frames. The closed bars indicate exons, bent lines show introns, and green arrows indicate predicted coding regions with the size shown in parentheses. The nucleotide positions with respect to TTV isolate TYM9 are shown in black.

By comparison, to VP1 of the CAV genome, it is thought that the protein expressed from ORF1 may code for the nucleocapsid of the virus with the arginine rich N-terminus involved with binding and encapsidation of the viral genome.

Several motifs are common to products generated by all known TTV isolates, including a putative Rep (replication associated) protein sequence thought to be involved with rolling circle replication, a C-terminus serine rich tract in ORF3, a conserved motif ($EX_8RX_2RX_{4-6}PX_{5-11}PX_{1-8}VX_1FX_1L$) in the C-terminus of ORF4 and a glutamine/glutamic acid rich domain in ORF1. TTV ORF2 also contains an amino acid motif ($Wx_7Hx_3Cx_1Cx_5H$) seen in the Circovirus CAV [Hijikata et al., 1999] as well as in TLMV, which again points toward a possible common ancestor for all three viruses.

Nucleotide sequence divergence resulting in amino acid substitutions is most notable in the central portion of ORF1. Three regions of marked amino acid conversion, of 22, 47 and 31 amino acids in length, in TTV genotype 1 have been designated HVR1 (hypervariable region 1), HVR2 and HVR3 [Nishizawa et al., 1999]. Divergence in this area results in the generation of quasispecies which have been observed in patients with non A-E hepatitis [Kurihara et al., 2001]

1.3.2 TLMV

The genomes of TLMV isolates range from 2850 – 2950 nucleotides in length and around 25% (~720 nucleotide) of this is untranslated, 5% less than the TTV genome. TLMV shares similar genomic organisation with TTV, but although four putative open reading frames have been predicted for some isolates (e.g. isolate CBD-231 used in Figure 1.3.1), many appear to have only ORF1 and ORF2 suggesting that ORF3 and ORF4 may not be functional. An initiation codon for the homologue to ORF3 in TTV has yet to be reported in any TLMV isolate [Biagini et al., 2001]. As with TTV, TLMV has a partially overlapping ORF1 and ORF2 and a conserved sequence located in the UTR. Although genomic sequences of TTV, TLMV and CAV contained very little similarity, there are conserved regions in the UTR which have facilitated phylogenetic comparison of the three viruses. This analysis confirms that TLMV is more closely related to TTV than to CAV however the authors propose that TLMV may be an intermediately related to TTV and CAV [Takahashi et al., 2000b]

The untranslated region of TLMV contains three direct repeats (DR1, DR2 and DR3) of a 27 nucleotide sequence. A 15 nucleotide portion of one of these direct repeats is also

found in isolation in the UTR of the prototype TTV sequence (TA278) [Takahashi et al., 2000b]. It has also been proposed that several highly conserved sequences in this non-coding region could become recombination breakpoints [Biagini et al., 2001] contributing to the genetic variability of this virus. The largest ORF is ORF1 which codes for 660 amino acids and, like the homologous protein in TTV, is thought to produce the viral glycoprotein because of the presence of an N-glycosylation site [Takahashi et al., 2000b]. The amino terminus of this protein is not only rich in arginine residues, but also tryptophan (W), in common with TTV but not CAV, and phenylalanine (F), in common with CAV but not seen in the ORFs of TTV. TLMV isolates also contain three of the conserved Rep associated motifs (FTL/FTxL, HxH and YxxK) related to rolling circle replication in ORF1 [Biagini et al., 2001]. TLMV ORF2 protein is predicted to be 90 amino acids in length and, as mentioned previously, contains the motif $Wx_7Hx_3Cx_1Cx_5H$.

1.4 Replication

It is still not known exactly how TTV and TLMV replicate however, the discovery of a Rep protein sequence suggests these viruses could replicate using rolling circle replication. Up to four amino acid motifs can form part of the Rep protein sequence which is conserved in many plant and animal viruses from the family *Circoviridae* [Niagro, 1998]. The TTV proteins appear to contain only two of the four motifs: the FTL and YXXK motifs have both been found in ORF1 however TTV, like CAV, appears to lack the P-loop motif which is thought to facilitate ATP/GTP binding. In CAV, the lack of this motif is thought to indicate the need for host topoisomerase or helicase activity to unwind the DNA strands for replication [Mushahwar et al., 1999; Niagro, 1998].

Investigation into the kinetics of chronic TTV viraemia in Hepatitis C virus (HCV) infected patients receiving interferon therapy estimated that 90% of TTV virions are cleared and renewed on a daily basis. A mathematical model predicted that at least 3.8 x 10^{10} virions must be produced daily in order to maintain an active infection [Maggi et al., 2001c].

TTV DNA has been detected in the nucleus of hepatocytes using in-situ hybridisation [Hu et al., 2002] and titres of the virus have been found at levels 10-100 times higher in bile than in peripheral blood [Ukita et al., 1999]. This lead to the conclusion that the main site of TTV replication was the liver, which would lend weight to the suggestion that the virus is cause of hepatitis and liver disease. Although double stranded replicative intermediates

of TTV have indeed been found in the liver, this is by no means the only site of viral replication. Double stranded forms of the virus have also been found in lymph node, muscle, thyroid gland, lung, spleen, pancreas and kidney [Bando et al., 2001; Okamoto et al., 2001a] and in liver, spleen, small intestine, lymphoid cells and bone marrow of experimentally infected rhesus monkeys [He et al., 2001; Xiao et al., 2002].

In 2000, Okamoto reported the detection of replicative forms of TTV in bone marrow cells but not in peripheral blood mononuclear cells (PBMCs) [Okamoto et al., 2000d]. A more recent study reported TTV DNA was present in PBMCs however replicative intermediates and mRNAs were only found in PBMCs stimulated with phytohemagglutinin, lipopolysaccharide and interleukin-2. TTV has also been shown to replicate in the human cell lines Chang liver and Raji, a B-lymphoblast cell line as demonstrated by infectious particles in the cell culture supernatant [Desai, 2005]. It is likely that TTV relies on the host cells replicative machinery to replicate and, like Parvoviruses, may require the host cell to be actively undergoing mitosis for viral replication to occur.

Although there is currently no data regarding the replication of TLMV, because of the presence of three of the four Rep associated amino acid motifs it is also thought to utilise rolling circle replication.

1.5 Transmission

The modes of transmission of TTV and TLMV are poorly understood, with different detection methods resulting in conflicting results. TTV was originally isolated following a blood transfusion, and the name Transfusion Transmitted virus was used in some publications. TTV has been detected in blood, blood products and PBMCs [Okamoto et al., 1999a; Pisani et al., 1999] and is found at a higher prevalence in populations subject to repeated blood transfusions or solid organ transplant [Gallian et al., 1999] as well as in groups at risk of parenterally transmitted viruses such as those with haemophilia [Chen et al., 1999], thalassemia [Prati et al., 1999] and on haemodialysis [Ali et al., 2004]. The infectious properties of TTV in blood derivatives was shown by successfully infecting chimpanzees using TTV-infected human serum [Tawara et al., 2000].

TTV can be found in over 90% of the general population which points towards routes of transmission other than parenteral. TTV has been detected in bile juices and faeces of viraemic individuals [Itoh et al., 2001] and viral particles isolated from faeces have been visualised by electron microscopy [Itoh et al., 2000] suggesting that the faecal-oral route of

transmission might be significant. TTV of faecal origin has been shown to be infectious both in tissue culture [Maggi et al., 2001b] and by experimental inoculation of rhesus monkeys using faecal filtrate [Luo et al., 2000]. A direct correlation between TTV infection and antibodies to enterically transmitted Hepatitis A virus, primarily transmitted by the fecal-oral route, has also been established [Saback et al., 1999]. Viral shedding of TTV in faeces is also likely to be the source of TTV which has been found in molluscs from the Norwegian coast [Myrmel, 2004] as well as in 97% of influent samples from wastewater treatment plants in Japan [Haramoto, 2005b] and in 5% of surface water samples collected from the Tamagawa River in Japan [Haramoto, 2005a]. Although it has yet to be established if TTV from these sources is infectious, this environmental contamination may account for a proportion of infections.

Initially TTV was thought to be acquired postpartum because virus was not detected in neonates born to infected mothers or in cord blood [Prati, 1999] however subsequent studies have found TTV and TLMV DNA both in cord blood, amniotic fluid and breast milk [Goto et al., 2000; Matsubara et al., 2001] implying that infection might also occur by vertical transmission from mother to infant either during or immediately after birth. Analysis of paired blood samples from Italian mothers and cord blood samples showed that they had similar titres of TTV thus making it unlikely the cord blood samples were contaminated by maternal blood [Morrica, 2000]. Sequence analysis of paired mother and infant blood samples demonstrated a varying proportion of shared homology between samples [Bagaglio et al., 2002; Lin et al., 2002]. This suggests that although intrauterine transmission of TTV is possible it does not appear to play a significant role. Even infants born to TTV negative mothers can acquire infection within the first couple of months of life and risk of infection increases with age [Ohto et al., 2002] so exposure to environmental factors seems to be more significant in the acquisition of TTV in childhood.

The role of sexual transmission of TTV has yet to be validated, although a study of individuals at high risk of sexually transmitted infection in Turkey failed to find an increased prevalence in these groups compared with the control group [Yazici et al., 2002]. TTV has been detected in saliva at higher levels than in corresponding serum samples leading to speculation it might be able to replicate in the salivary glands [Deng et al., 2000] and be transmitted via kissing. Both TTV and TLMV have been found in cervical swabs [Fornai et al., 2001] and TTV isolated from semen samples [Matsubara et al., 2000]

however, given the exposure to TTV in the environment and peri- and postnatally, it is unlikely that individuals reach sexual maturity without previous TTV infection.

1.6 Genetic heterogeneity

TTV has a remarkable range of sequence divergence for a DNA virus leading to speculation the replication machinery has little or no proofreading activity. This heterogeneity is unevenly distributed in the genome. The untranslated region is relatively well conserved with even divergent genotypes only differing by only 30% at the nucleotide level and some areas, which are presumed to be involved in translation or replication, displaying 95% nucleotide conservation. The coding regions, on the other hand, have regions that displayed up to 40% nucleotide sequence divergence [Erker et al., 1999] although the most sequence divergence appears to be located in three hypervariable regions situated in ORF1. Substitutions in this area result in the generation of circulating TTV quasispecies, similar to those seen in HIV and HCV [Nishizawa et al., 1999].

This diversity has inevitably led to problems with PCR based detection of TTV. The original N22 clone, which was used to generate PCR primers utilised in the initial prevalence studies, was a portion of the highly divergent ORF1. N22 primers preferentially amplify genotypes 1 and 2, so these data invariably underestimated the spread of the virus and do not give an accurate representation of the genotypes present *in vivo*. The generation of primers which amplify a region of the more conserved UTR greatly enhanced the detection capabilities of PCR based assays, however caution needs to be exercised when selecting primers and interpreting results as different pairs of primers, even when amplifying the same region, may not produce comparable results.

At least 39 genotypes of TTV have been described to date which differ from each other by >30% at the nucleotide level of the N22 region (ORF1) [Biagini et al., 1999]. These have been further classified into five major genetic groups that differ by at least 50% from one another: group 1 contains the prototype TTV (TA278) representing genotype 1 as well as genotypes 2-6. Genotypes 7, 8, 17 22 and 23 are representative of group 2. Group 3 comprises genotypes 9-16 and 18-20 as well as the eight SENV genotypes and group 4 contains genotypes 21 as well as nine novel genotypes isolated from infants in China and Japan. Group 5 contains a further four unidentified novel genotypes, again isolated from Chinese and Japanese infants [Peng et al., 2002].

Although TLMV was discovered two years after TTV, there are only 12 complete genome sequences submitted to GenBank. Analysis of these full length nucleotide sequences have shown that they can be classified into three genetic groups which differ from each other by >40% [Biagini et al., 2001]. In fact, 53 TLMV sequences cloned from a single French haemodialysis patient showed very high heterogeneity, with a genetic distance of 40.5% [Gallian et al., 2002]. These preliminary studies suggest that the TLMV genome is equally or even more divergent than the TTV genome.

Two studies have suggested that homologous recombination may occur frequently in both TTV and TLMV [Biagini et al., 2001; Worobey, 2000]. Evidence for recombination both between genotypes and among the same genotypes has been described with a large proportion of the recombination breakpoints for both viruses found in the relatively conserved UTR. Such events could contribute to the extensive heterogeneity of these viruses.

1.7 Epidemiology

1.7.1 TTV

TTV was initially isolated from an individual with post transfusion hepatitis in Japan, however the virus was quickly isolated from a number of different populations including blood donors [Charlton et al., 1998]. Epidemiological studies carried out since then have confirmed the global distribution of the virus [Prescott, 1998].

Early studies utilised the N22 PCR amplifying a sequence in ORF1. As described earlier, this limits the detection to genotypes 1 and 2 so data from these publications does not give a true representation of the global prevalence of these viruses. The development of the UTR PCR invariably increased the detection of TTV in geographical regions from less than 30% to over 70%, as shown in Table 1.7.1, however these primers may also bias the genotypes amplified.

Country	N22 primers	UTR primers	Reference(s)
UK	1.9%	77%	[Simmonds et al., 1998; Simmonds et al., 1999]
Holland	6%	72%	[Prescott et al., 1999; Simmonds et al., 1999]
Italy	10%	87%	[Zehender et al., 2001]
France	5%	75%	[Biagini et al., 1998; Biagini et al., 2000]
Finland	17%	73%	[Kakkola et al., 2004; Simmonds et al., 1999]
Egypt	29%	85%	[Abe et al., 1999; Gad et al., 2000]
Saudi Arabia	19%	100%	[Prescott et al., 1999; Simmonds et al., 1999]
Japan	23%	92%	[Takahashia, 1998]

Table 1.7.1 – Prevalence of TT virus in the general population of different countries as determined by PCR analysis using primers situated in ORF1 (N22 primers) and the non-coding region (UTR primers).

The continuation of research into the genetic variability of this virus will undoubtedly lead to the documentation of further genotypes, and may result in the discovery of near universal global infection.

1.7.2 SENV

Although there are eight SENV genotypes, designated A to H, the possible association between strains D and H and non A to E hepatitis have led to investigation of these strains dominating the research. Certainly, the incidence of SENV D and H infection is significantly higher in transfusion recipients who developed non A-E hepatitis than in those who had received a blood transfusion but did not subsequently develop hepatitis [Umemura et al., 2001b]

The distribution of these strains in blood donors varies geographically (Table 1.8.1) with 6.75% and 5% of blood donors infected with SEN D and SENV H respectively in China [Mu et al., 2004]. This is compared with 3% and 5% in Germany, 7% and 3% in Japan and 0.9% for both SENV D and SENV H in the USA [Shibata et al., 2001; Umemura et al., 2003].

Although there are relatively few studies investigating the prevalence of these viruses in Europe, it appears that the ratio of SENV D and SENV H in Europeans is different than in the Asian populations studied. The general population of China, Japan and Taiwan (as represented by blood donors and healthy controls) all have a higher proportion of SENV D than SENV H however in both Germany and Turkey the opposite is true (Table 1.8.1). This theory is investigated in chapter three.

1.7.3 TLMV

Very little is known about the epidemiology of TLMV. Prevalence studies have shown that 48% of sera from Norwegian blood donors and 72% of Brazilian blood donors are TLMV DNA positive [Moen et al., 2002a; Niel and Lampe, 2001]. As previously mentioned, TLMV may be more heterogeneous than TTV and the most sensitive PCR based systems rely on primers situated in the UTR of the genome. TTV and TLMV share a partially conserved UTR and care needs to be exercised to ensure that only the genome of interest is amplified.

1.8 Pathogenicity

TTV was originally isolated from a patients with post transfusion non A-G hepatitis [Nishizawa et al., 1997] at titres which correlated with ALT levels. This led researchers to hypothesise that TTV was responsible for cases of unexplained chronic and acute hepatitis however further studies produced data which cast doubt on this. Of most note was the finding that TTV was highly prevalent in the general population, with one study reporting that TTV was detected in 92% of individuals in Japan [Takahashia, 1998]. Comparison of the prevalence of TTV in healthy controls and in those with cryptogenic hepatitis showed there were no significant differences [Fukuda et al., 1999; He et al., 1999] and there was also no association between ALT levels and TTV positive and negative individuals [Fukuda et al., 1999; Takahashia, 1998].

Table 1.8.1 – Prevalence of SENV D and SENV H in different populations within various countries. SENV D+H column indicates prevalence of individuals simultaneously infected with both SENV D and SENV H.

Geographical location	Patient group status	SENV D prevalence	SENV H prevalence	SENV D+H prevalence	Reference
China	Acute HAV	3.6%	21%	11%	[Mu et al., 2004]
	Chronic HBV	13%	12%	32%	
	Chronic HCV	20%	20%	45%	
	Acute + chronic non A-E hepatitis	24%	17%	27%	
	Blood donors	6.7%	5%	19%	
Germany	Blood donors		16.8%		[Schroter, 2002]
	IVDUs (HIV-)		23%		
	Haemodialysis patients		12.8%		
	Haemophiliacs		34%		
	Transfusion recipients		30%		
	HIV+		44%		

	HCV+		28%		
Germany	Blood donors	3%	5%	0%	[Umemura et al., 2003]
Greece	Blood donors	16%	7%	1%	[Umemura et al., 2003]
Italy	HIV+	5.2%	1.7%		[Quiros-Roldan, 2003]
Japan	Blood donors	14%	2%	7%	[Umemura et al., 2003]
Japan	HAV+	7%	0%	0%	[Umemura et al., 2003]
	HBV+	10%	5%	5%	
	HCV+	21%	5%	3%	
	Non-viral liver disease	17%	3%	2%	
	Healthy controls	18%	4%	1%	
Japan	Fulminant hepatitis	60%	0%		[Sugiura et al., 2004]
	Acute hepatitis	5%	5%		
	Chronic hepatitis	11%	0%		
	Blood transfusion recipients	17%	7%		
	Control	13%	2%		

	Pregnant women	15%	12%		
Taiwan	IDU s	46%	12%	4%	[Kao et al., 2002]
	Haemophiliacs	68%	12%	12%	
	Thalassemic	85%	25%	17%	
	Haemodialysis patients	68%	22%	22%	
	Non A-E fulminant hepatitis	30%	0%	0%	
	HBV +	40%	12%	6%	
	HCV+	57%	28%	15%	
	Healthy adult	15%	2%	2%	
Thailand	Blood donors			5%	[Tangkijvanich et al., 2003]
	Chronic liver disease			25%	
	Hepatocellular carcinoma			42%	
Turkey	High ALT	5%	8%	13%	[Serin et al., 2005]
	Healthy individuals	4%	6%	10%	

USA	Hepatitis associated aplastic anemia	8%	19%	8%	[Umemura et al., 2003]
USA	Blood donors	0.9%	0.9%	0%	[Umemura et al., 2003]
USA	IVDUs	33%	38%		[Wilson et al., 2001]
USA	Transfusion recipients			30%	Umemura, 2001 #1423}
	No transfusion			3%	
	Blood donor			1.8%	

It was also shown that acquiring TTV following blood transfusion was not associated with the development of liver disease or an increase in ALT levels [Gimenez-Barcons et al., 1999; Matsumoto et al., 1999]. This evidence suggests that TTV is unlikely to be pathogenic *per se*, however the heterogeneous nature of TTV means that some of the genotypes may be involved in a disease process. The prevalence of TTV in the general population frequently leads to viral co-infections and transmission of TTV during blood transfusion may lead to the introduction of TTV or novel genotypes of TTV in individuals with underlying conditions. Zein and co-workers studied a cohort of HCV positive patients with well characterised disease and found TTV to be significantly more prevalent in those with advanced liver disease (decompensated cirrhosis and hepatocellular carcinoma) compared to those with stable liver disease (chronic hepatitis and compensated cirrhosis). Univariate analysis showed that TTV was important for predicting the stage of liver disease in HCV positive individuals [Zein et al., 1999]. Another study investigating a group of patients with fulminant hepatic failure discovered 100% of those with TTV died compared with only 47% of those who were negative for TTV DNA [Tanaka et al., 1999]. Other investigator's findings failed to support this and reported that TTV infection has no impact on disease progression in HCV positive patients [Kao et al., 2000; Tangkijvanich et al., 1999].

Histopathological analysis of liver tissue taken via percutaneous needle biopsy from TTV positive and negative non-B, non-C and non-G chronic hepatitis patients showed mild fibrosis and periportal/piecemeal necrosis which were not seen in the TTV negative control group [Suzuki et al., 2001]. Hu also found mild pathogenicity when TTV DNA positive liver biopsy samples from patients suffering from non A-G hepatitis were studied [Hu et al., 2002].

During experimental inoculation of hepatocyte cell lines with TTV genotype 1 to generate an *in vitro* replication system, infection of a Chang liver cell line (derived from a non malignant liver tissue) produced morphological changes in the cells. The initial cytopathic effect (CPE) occurred 2-3 days post inoculation and was characterized by focal cell rounding and granulation. By day six the entire cell layer had degenerated. This suggests that TTV genotype 1 may be able to cause cell damage in the liver of infected individuals when unrestricted replication of the virus occurs [Desai, 2005]. Interestingly, although a phytohemagglutinin stimulated TTV negative PBMC cultures and a B lymphoblast cell

line were also shown to support active TTV replication in the same study, no morphological changes were observed in these cells.

The possible pathogenicity of TTV was also investigated in research which compared the apoptotic potential of a protein encoded by ORF3 of TTV with Apoptotin, a protein produced by CAV which has been documented to cause apoptosis solely in cancer cell lines. The 105 amino acid protein from TTV genotype 1, putatively named TTV-derived apoptosis-inducing protein (TAIP), was shown to cause apoptosis in a hepatocellular carcinoma (HCC) derived cell line at a significantly higher level than in a non-HCC cell line [Kooistra et al., 2004]. Unfortunately, the effect of the TAIP construct on normal liver cells was not determined but these data do appear to suggest a pathological role for TTV, at least in relation to HCC.

The pathogenicity of TTV in mice was examined in vivo by creating transgenic mice using a gene expression cassette which contained ORFs 1, 2-4, 2-5, 3 and 6 from TTV genotype 1. Of the 84 mice born, eight were transgenic and contained a mixture of the ORFs in the constructs. One of the mice produced offspring, which contained only ORF1 and had a phenotype of reduced growth, retardation and premature death from ascites. These mice had high levels of gene expression in the kidneys and had pathological changes in this tissue which resembled nephrotic syndrome in humans. There were no abnormalities seen in the liver of any of the transgenic mice [Yokoyama et al., 2002].

Transmission studies have also been used to try to assess the pathogenicity of TTV. Mushahwar used human serum from patients with chronic non A-E hepatitis and TTV infection to inoculate chimpanzees however no histological of biochemical evidence of hepatitis or liver disease was seen [Mushahwar et al., 1999]. In a separate experiment, two chimpanzees were infected with human TTV extracted either from feces or from serum. One chimpanzee had detectable TTV DNA in its serum from week five to week 15 post inoculation (p.i.). An increase in ALT levels, which corresponded to mild biochemical and histological changes in the liver, was seen just before the virus was cleared at week 15. The second chimpanzee became viraemic at week seven p.i. but had failed to clear the infection at week 30 when observation ceased. A peak in ALT levels was seen in week 16 which again corresponded with mild ballooning and degeneration of hepatocytes [Tawara et al., 2000]. Both these experiments suggest that TTV, or at least TTV genotype 1, may be pathogenic.

The finding that TTV was not solely a hepatotropic virus led to an increase of investigations into the role of TTV in extrahepatic diseases or complications of liver diseases which are not directly related to liver function. Using the N22 PCR which detects TTV genotype 1, platelet count in patients with chronic hepatitis C infection and chronic hepatitis with no markers of HCV or HBV was found to be lower in those who were TTV DNA positive compared with the TTV negative group [Tokita et al., 2001]. A further study compared platelet counts in chronic hepatitis C patients before and after treatment with interferon and found that individuals who had concomitant TTV infection with genotypes 1-4 had significantly increased thrombocytopenia both before and after interferon treatment [Tokita et al., 2002]. TTV has also been implicated in aplastic anemia following post transfusion hepatitis [Kikuchi et al., 2000] however other investigators [Poovorawan et al., 2001; Udomsakdi-Auewarakul et al., 2000] have refuted this.

TTV has also been implicated in the exacerbation of autoimmune disorders such as multiple sclerosis (MS) and systemic lupus erythematosus (SLE). Investigation of *in vivo* clonally expanded T-cells isolated from the cerebrospinal fluid of an MS patient during relapse showed that peptides from the arginine rich N-terminus of TTV ORF1 could stimulate autoreactive T cell clones which may lead to disease exacerbation [Sospedra, 2005]. In patients with SLE, not only was TTV found to be significantly more prevalent than in blood donors, but TTV was thought to generate antibodies cross reactive to a human endogenous retrovirus nuclear protein, an autoantigen associated with SLE [Gergely, 2005].

SENV, like TTV, has been extensively investigated to establish conclusively a link between the virus and cryptogenic hepatitis. SENV D and SENV H in particular have been found in a significantly higher percentage of patients with liver disease as well in those with a history of frequent blood transfusion and intravenous drug users than in healthy, low risk populations (Table 1.8.1). There seems to be little doubt that SENV D and H are more prevalent in high risk groups but no evidence that they are involved in causing hepatitis directly or in increasing the severity of hepatitis caused by HCV or HBV [Kao et al., 2002; Umemura et al., 2003].

TLMV is still very much an unknown quantity with, at the time of writing, only 17 papers published on this virus. As yet there has been no research investigating the pathogenesis of TLMV.

1.9 Immune responses

The most characteristic feature of TTV and TLMV is their ability to produce chronic infection, although little is known about the mechanisms of persistence. The ubiquity of the virus and the prevalence in the general population has led some researchers to hypothesize that TTV may have a commensal relationship with humans [Simmonds, 2002]. It is also possible that infection either in utero or peripartum leads to a degree of immune tolerance as seen with hepatitis B virus (HBV).

Sequence analysis of TTV genotype 1 isolated from patients with chronic and acute TTV infections showed that those with chronic infection had a high percentage of substitutions in the hypervariable region of ORF1. This resulted in the generation of quasispecies which may help the virus escape immune surveillance. These quasispecies were not seen in acutely infected individuals who also had a higher proportion of free TTV compared with IgG complexed TTV as determined by immunoprecipitation with goat anti-human IgG followed by PCR of both the precipitate and supernatant. On the other hand, immune complexes tended to be frequent in chronically infected patients, by far outweighing any free virus present [Nishizawa et al., 1999]. Immune complexes of virus with IgG are discussed in chapter five of this thesis.

Ott and colleagues carried out a western blot using a recombinant C-terminus protein from ORF1 and serum from French blood donors, healthy children and patients with cryptogenic hepatitis. A positive serological response (anti TTV IgG + IgA + IgM) was determined in 98.7%, higher than the 76.1% who were PCR positive. Further analysis demonstrated that none of the sera tested were positive for anti-TTV IgM, therefore none of the viraemia was the result of novel infections [Ott et al., 2000]. The authors suggested that the C terminus of ORF1 was an immunodominant epitope, likely to be highly antigenic in vivo.

IgM class antibodies have been detected in two patients with post transfusion non A-G hepatitis and in an individual with acute TTV infection. The time from TTV DNA detection in the serum of these individuals and IgM production was between 2-7 weeks. In one patient, anti-TTV IgG was detected after IgM had disappeared from circulation but in the other 2 individuals, both IgM and IgG were present simultaneously [Tsuda et al., 2001]. In a second study, IgM class antibodies against a recombinant protein generated from ORF2a and ORF2b of TTV genotype 6 were detected in 9% of healthy individuals. Interestingly, anti-TTV IgM against the product of ORF2b were detected over a number of

years during follow up, with and without the presence of TTV DNA in serum. It is as yet unknown why the IgM phase of immune response persisted in these individuals. Only 10% were IgG antibody positive to the same antigens, with all of these individuals also being TTV DNA positive [Kakkola et al., 2004].

Approximately 80-95% of TTV isolated from the sera of HIV positive individuals with high TTV DNA titre is complexed with IgG, however TTV in faeces is usually in the form of uncomplexed free virus. Electron microscopy of TTV infected sera visualised aggregates of TTV with IgG. An aggregate of TTV particles could be visualised in fecal supernatant only on the addition of γ-globulin which contained TTV-specific antibodies [Itoh et al., 2000].

These data conclusively prove that some individuals are capable of eliciting both an IgM and an IgG immune response to some of the proteins encoded by the TTV genome. It is unknown if these antibodies are cross protective, however the presence of TTV DNA simultaneously with antibodies suggests they might not be [Kakkola et al., 2002]. From these limited data, it is difficult to ascertain if the paradigm of IgM antibody in early infection followed by IgG can be applied to these viruses.

There is evidence that TTV and TLMV titres in the serum of immunosuppressed individuals are raised. Soldiers undergoing an intensive week long training program known to cause physical and mental exhaustion and shown to impair their immune systems (30% decrease in circulating lymphocytes and decreased immunoglobulins) had an increase in TTV and TLMV titre however the change was not statistically significant. Patients subject to immunosuppressive treatment following kidney transplant experienced a significant increase in TTV and TLMV viral loads [Moen et al., 2003]. This is corroborated by increased TTV titres in liver transplant recipients who were also taking an immunosuppressive drug regime [Shang et al., 2000]. The change in TTV and TLMV titre in relation to the immunosuppression associated with HIV infection and AIDS is discussed in chapter four.

The role of the immune system in the control of TTV viraemia is further supported by reports that stimulation of the immune system with interferon for the treatment of HCV reduced the TTV titre or completely cleared the virus in the majority of cases [Maggi et al., 2001c; Nishizawa et al., 2000]. In many cases, however, this decrease in circulating TTV was not maintained [Maggi et al., 1999; Maggi et al., 2001c]. Touinssi also discovered the

apparent increased prevalence of TTV in haemodialysis patients and diabetics actually reflected an increase in elevated viraemia in these populations, resulting in an increase in patients whose viraemia was above the detection limit of the assay. Haemodialysis patients and diabetics can display a degree of immunosuppression, which could act as an explanation for this phenomenon [Touinssi et al., 2001].

1.10 HIV/AIDS and Anelloviruses

Acquired Immunodeficiency Syndrome (AIDS) was first described in 1981 following an outbreak of immunodeficiency related opportunistic infections among homosexual men in the Los Angeles area of the United States [CDC, 1981]. In 1982, similar symptoms were observed in further populations including haemophiliacs, blood transfusion recipients and the babies of high-risk mothers, which led scientists to believe they were looking for a pathogenic agent transmitted via blood and body fluids. By 1984, scientists had isolated a candidate Retrovirus which was later named Human immunodeficiency virus (HIV) [Barre-Sinoussi et al., 1983].

1.10.1 HIV virion

HIV is a member of the genus Lentivirus, from the *Retroviridae* family of non-oncogenic viruses infecting humans, non-human primates and some domesticated mammalian species [Ratner et al., 1985]. It is an icosahedral virus (Figure 1.10.1), 80-110nm in size, with a lipid envelope that is largely derived from the host cell plasma membrane. This lipid bilayer is spiked with the two main viral envelope proteins: glycoprotein (gp) 120 protrudes from the virus and is essential for the attachment of the virus to the CD4 receptor on host cells, and the transmembrane protein gp41 which spans the lipid bilayer and facilitates the fusion of the viral and cell membranes.

The capsid encapsulates two copies of single stranded RNA as well as reverse transcriptase and other enzymes needed for viral replication. The nine kb of viral genome contains nine genes (Table 1.10.1): structural genes gag, pol and env, transactivation genes rev and tat, and accessory genes nef, vpu and vif [Hirsch et al., 1996; Varmus et al., 1998].

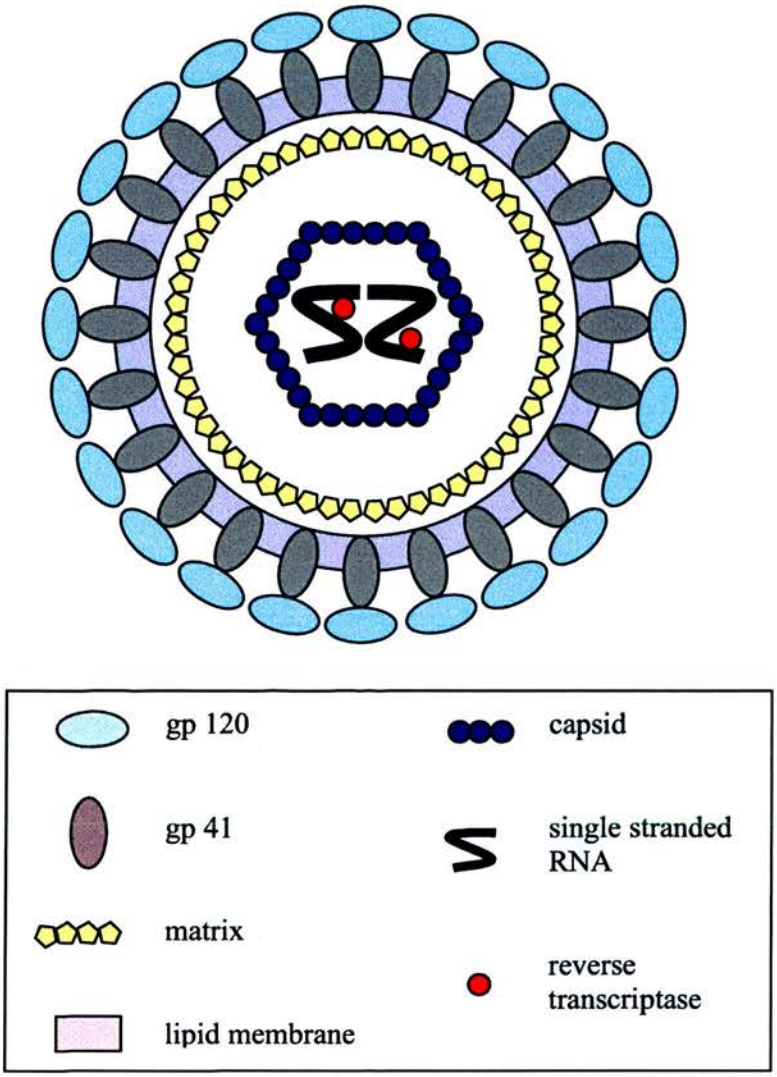

Figure 1.10.1 - Diagram of HIV virion.

1.10.2 HIV life cycle

The HIV life cycle begins with the viral glycoprotein gp120 binding with high affinity to the CD4 receptor on the surface of the host cell T-lymphocyte or macrophage. Co-receptor binding is also needed for viral entry into the cell, for X4 strains of HIV the chemokine receptor CXCR4 is used, and R5 strains of HIV use the receptor CCR5. Once receptor and co-receptor binding has occurred glycoprotein gp41 facilitates the fusion of the viral envelope and the cell membrane allowing viral RNA, gag proteins and pol enzymes to enter the cell cytoplasm [Chan et al., 1998; Dimitrov et al., 1997].

HIV Gene	Function of gene
gag (group specific antigen)	encodes virus core proteins: p24 - capsid protein p17 - matrix protein p9 - binds to viral RNA p7 - binds to viral RNA
pol (polymerase)	encodes enzymes: reverse transcriptase - converts RNA to DNA integrase – integrates virus into host cell DNA protease – cleaves non-functional core precursor proteins
env (envelope)	encodes virus envelope proteins: gp120 – mediates binding of virus to CD4 receptor on host cell gp41 – fuses virus with cellular membrane
tat (transactivator)	encodes Tat protein – upregulates viral transcription
rev (regulator of viral expression)	encodes Rev protein - regulates viral RNA transport and splicing
vif (viral infectivity factor)	encodes Vif protein – facilitates virion maturation
vpu (viral protein U)	encodes Vpu protein – promotes virus budding
nef (negative-regulation factor)	encodes Nef protein – interacts with host cells inducing longevity in infected cells and apoptosis in non infected cells
vpr (viral protein R)	encodes viral protein R – regulates gene expression and induces cell cycle arrest

Table 1.10.1 – Function of HIV genes.

The viral reverse transcriptase then catalyses the production of double stranded DNA from the single stranded RNA and this migrates to the nucleus of the cell where it integrates with the host DNA to form a provirus. Once the provirus has been inserted into the genome the only way the virus can be eliminated is by destroying the cell itself [Hirsch et al., 1996].

Once integrated, the provirus can either remain latent or use the host cell's RNA polymerase II to produce RNA transcripts, which are spliced into mRNAs that are transported into the cytoplasm to be translated into regulatory or precursor proteins. The precursor core proteins are cleaved by the viral protease and components for a new virion assemble and move towards the cell's surface, pinching off from the cell to form an infectious virus. Virus can also be released from an infected cell when the cell lyses [Hirsch et al., 1996].

1.10.3 Pathogenesis of HIV infection

Primary infection with HIV is usually characterised by 'flu like symptoms, lymphadenopathy, rash and weight loss, which is the result of the virus replicating unchecked by the immune system and disseminating throughout the body. The virus also forms reservoirs of infection in the lymph nodes and gut associated lymphoid tissue [Brenchley et al., 2004]. The high viral load stimulates the production of HIV specific CD4+ T lymphocytes to control the infection however viral protein gp120 has a high affinity for the CD4 receptor and these cells are quickly infected and destroyed. As the production of cytotoxic CD8 T lymphocytes, which kill the infected cells, and the trapping of the virus in follicular dendritic network of the lymph nodes decreases the amount of virus in the bloodstream and as the viral load decreases, the CD4 count rises towards the pre infection level (Figure 1.10.2)[Fauci et al., 1993].

After primary infection and seroconversion, a state of dynamic equilibrium occurs between the numbers of cells becoming infected, the number of CD4+ T lymphocytes being produced and dying, and the level of viral replication. This chronic stage of HIV infection lasts on average ten years and during this time there is usually a slow progressive increase in viral load and decrease in CD4 count as the immune response becomes less and less effective against the virus. The rate of disease progression appears to be dependent on the success of the virus adapting in response to evolutionary pressures [Fauci et al., 1996].

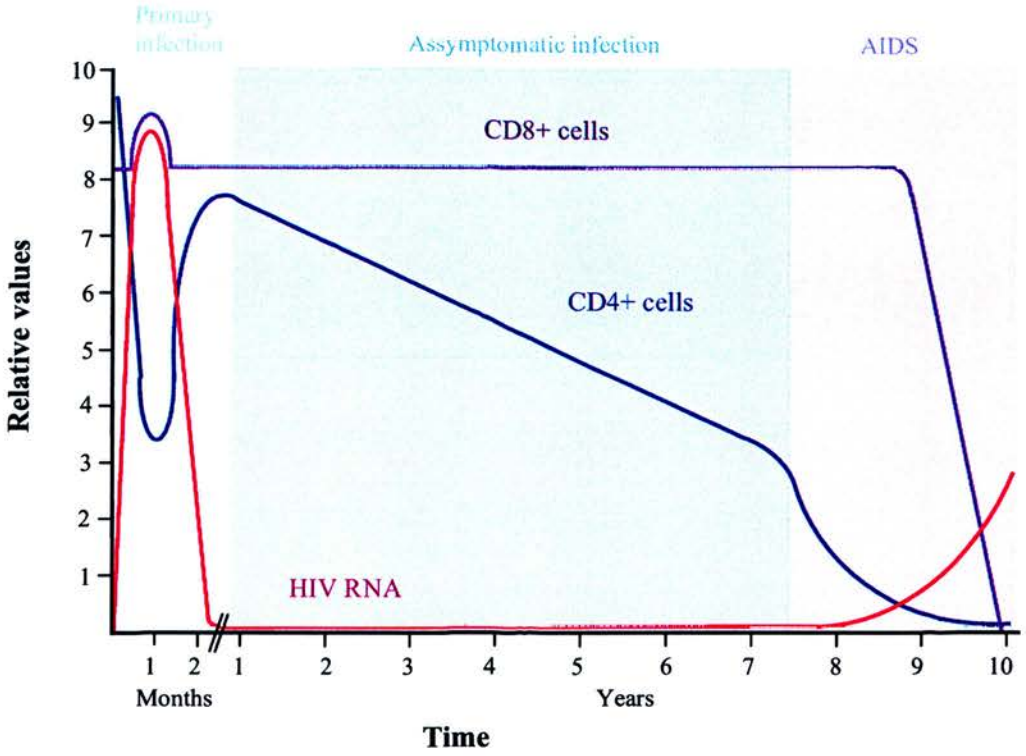

Figure 1.10.2 – HIV titre, CD4+ and CD8+ T lymphocyte numbers during stages of HIV infection.

Initially the R5 strain of the virus, a non-syncytial forming strain which uses the chemokine receptor CCR5 as the coreceptor, predominates but a shift towards strains that use the coreceptor CXCR4 and have the ability to form syncytia tends to occur over time. This change of the predominant virus population allows the virus to kill CD4+ T lymphocytes which are not directly infected and is responsible for a more rapid decline of CD4+ cells and a faster progression towards AIDS [Connor et al., 1993].

AIDS is confirmed when the CD4 count falls to less than 200 cells per μl or by the presence of one of 25 conditions which indicate severe immunodeficiency such as pneumocystis carinii pneumonia and toxoplasmosis.

1.10.4 Effect of HIV on the immune system

The predominant cells infected and destroyed by HIV are CD4+ T lymphocytes but the impact that the virus has on the immune system is not restricted to these cells. CD4+ T cells also play a pivotal role in the induction CD8 T and B cells which are crucial for an effective immune response. A summary of effects the reduction of CD4+ lymphocytes has on the immune system is outlined in Figure 1.10.3. The type of immune deficiency associated with the depletion of CD4+ T lymphocytes and the direct and indirect effects this has on the immune system leaves the patient vulnerable to infections by fungi, protozoa, bacteria and viruses. It is this central role in immune control that makes the CD4 count the most accurate way of assessing disease progression and therefore the level of immunodeficiency [Hirsch et al., 1996].

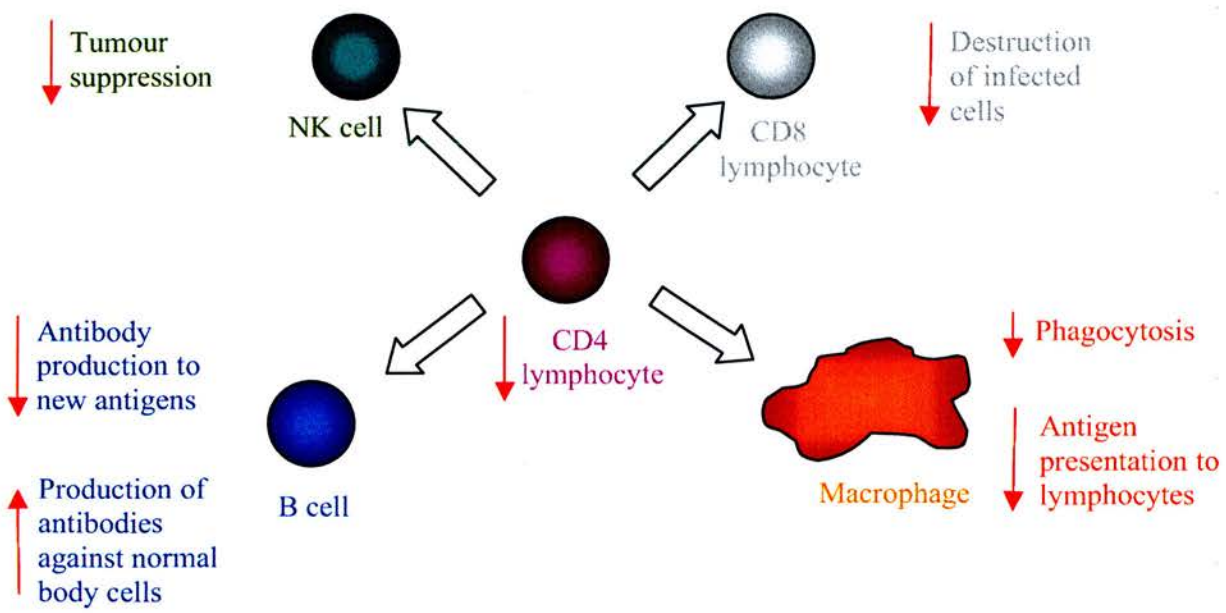

Figure 1.10.3 – Effect of CD4+ T lymphocyte depletion on immune system.

CD8 T lymphocytes are also critical to the immune system and the sudden depletion of these cells often precedes the decline into AIDS. Unlike CD4+ cells, CD8 cells are not the primary targets for the virus although some CD4+CD8+ cells have been documented which would be susceptible to infection [Imlach et al., 2001]. CD8 lymphocytes express

the coreceptor CXCR4 and it appears that the syncytia forming strain which dominates the latter stages of infection can bind to this ligand and induce apoptosis in these cells leading to a sharp decline in cell numbers [Herbein et al., 1998; Livingstone et al., 1996].

1.10.5 HIV and concomitant viral Infections

The immunodeficiency associated with HIV infection and AIDS results in opportunistic infections (OIs) by pathogens that would usually cause a mild or asymptomatic infection in immunocompetent individuals. These usually occur after the CD4 count has fallen below 500 cells per μl and signify the progression towards full-blown AIDS.

Similar routes of transmission often result in HIV positive individuals harbouring concomitant viral infections, for example up to 80% of intravenous drug users and 98% of haemophiliacs are also infected with HCV [Mohsen et al., 2002]. These co-infections can have both positive and negative effects on the disease outcome for both HIV and the second virus.

Although still not conclusively proven, there are some studies indicating that HCV infection has an adverse effect on HIV pathogenesis and can speed the progression towards AIDS [Greub et al., 2000]. HIV also results in an increased HCV viral load and a more rapid onset of liver disease, possibly due to the depletion of CD4+ T lymphocytes [Valdez et al., 2000].

Conversely, GBV-C has been reported to have a beneficial effect on disease progression [Cainelli et al., 2001]. It was first described in 1995 and shares routes of transmission with HIV making co-infections common. The beneficial effect on HIV prognosis only occurs before seroconversion, during which viral clearance occurs. The presence of IgG antibody to GBV-C (usually with the absence of any detectable GBV-C RNA) is accompanied by a negative effect on HIV disease progression and a worse prognosis than seen with GBV-C uninfected individuals [Williams et al., 2004].

1.10.6 TTV/TLMV and HIV

No pathological role for TTV/TLMV infection has been established, however the high prevalence of virus in the general population leads to a high level of co-infections with other viruses. The immunodeficiency associated with HIV can result in opportunistic

infections and the reactivation of latent viruses so the natural history of TTV/TLMV in HIV positive individuals is of particular interest.

The role of TTV/TLMV in HIV infection and the progression towards AIDS is still uncertain with many studies publishing conflicting results on the significance of co-infection. A Danish HIV cohort had significantly higher prevalence and titre of TTV compared with blood donors (76% vs. 7%) [Christensen et al., 2000] however the number of blood donors testing positive for TTV appears to be particularly low. This is probably due to the use of PCR primers sited in ORF2 of the TTV genome which would restrict the genotypes of virus amplified. HIV positive patients tend to be infected with a higher number of mixed infections than blood donors [Devalle and Niel, 2004; Sherman et al., 2001] so PCR amplification of the most prevalent genotype is more likely in those with HIV. Further analysis of the HIV positive cohorts showed a significant inverse correlation between TTV titre and CD4+ T cells [Christensen et al., 2000; Shibayama et al., 2001] and high serum TTV and SENV H concentrations were also cited as an independent factor associated with decreased survival [Christensen et al., 2000; Sagir et al., 2005].

These studies appear to indicate a role for the immune system in the control of TTV/TLMV replication however a study following changes in TTV and TLMV viral load in individual samples failed to find a significant increase in titre following progression to AIDS or in conjunction with changes in CD4+ and CD8+ T lymphocytes counts [Moen et al., 2002b].

The high number of co-infections of HIV and HCV and the high prevalence of TTV in HIV positive populations invariably also results in HIV, HCV and TTV co-infections. Although it is now thought to be unlikely that TTV is the cause of non A-E hepatitis, there is evidence that infection with TTV combined with dual HIV and HCV infection increases the ALT levels in patients indicating more severe hepatitis than in patients infected only with HIV and HCV [Shieh et al., 2003] and with TTV and HIV [Sherman et al., 2001].

1.11 TTV-like viruses in non-human primates

Soon after TTV was discovered in humans, research to investigate TTV homologues in a number of non-human primate and animal species began. Romeo and colleagues used UTR PCR to amplify TTV from the serum and faeces of naïve chimpanzees (*Pan troglodytes*) bred in captivity in the USA. They found a high proportion of sera (65%) were PCR positive with sequences which were significantly different from published

human sequences, appearing to rule out transmission from human contact [Romeo et al., 2000]. TTV sequences from chimpanzees kept in captivity in The Netherlands and in a separate US facility showed 85-91% nucleotide homology and 93-96% homology at the amino acid level to the sequence published by Romeo, suggesting a chimpanzee specific TTV [Verschoor et al., 1999]. This was refuted by a study which found sequences isolated from chimpanzees, owl monkeys (*Aotus trivirgatus*) and tamarins (*Sanguinus labiatus*) as well as from farm animals were not genetically dissimilar from human sequences [Leary et al., 1999]. The primers utilized in this study annealed to the relatively non-conserved N22 coding region of the genome, known in humans to be an unreliable detection system because of the heterogeneity in this region.

TTV-like sequences have also been isolated from chimpanzees, Japanese macaques (Macacca fuscata), red-bellied tamarins (*Saguinus labiatus*), cotton top tamarins (*Saguinus oedipus*) and owl monkeys (*Aotus trivirgatus*) using UTR primers (Table 1.11.1). Sequences analysis showed that the UTR sequences, usually highly conserved (85-100% similar) within all human genotypes, did not show a high degree of conservation when compared with non-human primate sequences. Comparison of the prototype human TTV sequence (TA278) with nucleotide sequences from chimpanzees, macaques, tamarins and owl monkeys showed similarities of 66-90% similarity (chimpanzee), 62-72% similarity (macaque) and up to 57% similarity for both tamarin and owl monkey. Only TTV from chimpanzees was detectable using the N22 primers, suggesting that none of the TTV-like viruses infecting the Old World monkeys were sufficiently similar to the human strains in the ORF1 coding region to be amplified using this primer set [Okamoto et al., 2000a].

As with human TTV, the virus amplified from chimpanzee is extremely heterogeneous. At the present time, at least four genetic groups have been identified, differing by at least 30% at the nucleotide level in the N22 region. Phylogenetic analysis has revealed that chimpanzee sequences are not clustered in a separate group from the human sequences, rather chimpanzee and human sequences are interspersed [Okamoto et al., 2000a]. The possibility of human TTV infecting chimpanzees has been shown by transmission experiments however there has yet to be any incidence of chimpanzee TTV detected in humans [Mushahwar et al., 1999; Okamoto et al., 2000a].

Species	TTV positive serum (%)	Reference
Chimpanzee (Pan troglodytes)	98%	[Abe et al., 2000]
Japanese macaque (Macaca fuscata)	90%	[Okamoto et al., 2000a]
Red bellied tamarin (Saguinus labiatus)	100%	[Okamoto et al., 2000a]
Cotton-top tamarin (Saguinus oedipus)	83%	[Okamoto et al., 2000a]
Owl monkey/Douroucouli (Aotus trivirgatus)	100%	[Okamoto et al., 2000a]
Crab eating monkey (Macaca fascicularis)	14%	[Abe et al., 2000]
Tupaia (Tupaia belangeri chinesis)	26%	[Okamoto et al., 2000a]
Dog (Canis familiaris)	38%	[Okamoto et al., 2002]
Cat (Felis catus)	43%	[Okamoto et al., 2002]
Pig (Sus domesticus)	82%	[Okamoto et al., 2002]

Table 1.11.1 – Detection of TTV-like viruses in non-human primates and domesticated animals by PCR.

A TTV-like virus has also been detected in tupaias (tree shrews), which have characteristics of both primates and insectivores. Although the genomic organisation and transcription profile of the isolated viruses are analogous to the previously characterised TTV and TLMV, the genome differs from these by more than 50% [Okamoto et al., 2001b].

The tupaia virus has a small genome, more comparable in size to the animal viruses CAV and BFDV than to human TTV or TLMV (Table 1.11.2) however phylogenetic analysis showed the tupaia virus was closer to TLMV isolated from humans than from the TTV-like viruses infecting tamarins and owl monkeys [Okamoto et al., 2001b].

Species	Virus	Genome size (nucleotides)	Reference
Human (Homo sapiens)	TTV	3852	[Kamahora et al., 2000]
Human (Homo sapiens)	TLMV	2915	[Takahashi et al., 2000a]
Chimpanzee (Pan troglodytes)	TTV-like	3690	[Okamoto et al., 2000b]
Japanese macaque (Macaca fuscata)	TTV-like	3798	[Okamoto et al., 2000a]
Cotton-top tamarin (Saguinus oedipus)	TTV-like	3371	[Okamoto et al., 2000a]
Owl monkey/Douroucouli (Aotus trivirgatus)	TTV-like	3718	[Okamoto et al., 2000a]
Dog (Canis familiaris)	TTV-like	2797	[Okamoto et al., 2001b]
Cat (Felis catus)	TTV-like	2064	[Okamoto et al., 2001b]
Pig (Sus domesticus)	TTV-like	2878	[Okamoto et al., 2001b]
Chicken (Gallus domesticus)	CAV	2319	[Noteborn, 1991]

Table 1.11.2 – Comparison of genome sizes of Anelloviruses infecting humans, non-human primates and domesticated animals.

As well as primates, species-specific TTV-like viruses infect domesticated cats, dogs and pigs. Like tupaia TTV, virus genomes from cats, dogs and pigs are smaller than human TTV (Table 1.11.2) and phylogenetic analysis showed these three novel viruses have less than 45% homology with previously reported TTV and TLMV genomes as well as differing from each other by 54% at the nucleotide level. However, even in these genetically distant viruses, regions of homology with human and non-human primate TTV and TLMV were found, most notably two conserved 15 nucleotide sequences located in the UTR [Okamoto et al., 2002]. Further investigation of pigs from a herd in Brazil discovered a single swine infected with two strains of TTV which displayed only 45% homology, suggesting that swine TTV is as heterogeneous as its human counterpart [Niel et al., 2005].

Aims

The project had the following aims:

- to evaluate the prevalence of Anelloviruses in the Scottish blood donor population
- to investigate Anelloviruses in non-human primates
- to establish if domesticated farm animals are infected with Anelloviruses
- to attempt to clarify the role of the immune system in Anellovirus infection

Chapter 2

Materials and Methods

2 MATERIALS AND METHODS

2.1 Suppliers

Unless otherwise stated, all the chemicals used were supplied by Sigma-Aldrich. Oligonucleotide primers and digoxigenin labelled probes were synthesised by Operon.

Company	Address
Amersham	Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA
Anachem	Anachem House, Charles Street, Luton, Bedfordshire, LU2 0EB
BDH	McQuilkin & Co., 21 Polmadie Avenue, Glasgow, G5 0bb
bioMérieux UK Ltd.	Grafton Way, Basingstoke, Hampshire, RG22 6HY
Camblab	Camlab House, Norman Way Industrial Estate, Over, Cambridge, CB4 5WE
Dynal Biotech	Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF
Eppendorf-Netheler-Hinz GmbH	D22331 Hamburg, Germany
Kodak	Anachem, D'Arcy Way, LLandarcy, Neath, SA10 6EZ
National Diagnostics	Fisher Scientific Ltd. Biship Meadow Road, Loughborough, LE11 5RG
Operon Biotechnologies GmbH	Nattermannallee 1, D50829, Cologne, Germany
PerkinElmer	Chalfont Road, Seer Green, Beaconsfield, Bucks, HP9 2FX
Promega UK Ltd.	Delta House, Chilworth Science Park, Southampton, SO16 7NS
Qiagen	Qiagen House, Fleming Way, Crawley, West

	Sussex, RH10 9NQ
Roche Diagnostics Ltd.	Bell Lane, Lewes, East Sussex, BN7 1LG
Sigma-Aldrich	The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT

Table 2.1.1 – Contact information for suppliers used to source reagents for this thesis.

2.2 Reagents

Reagent	Constituents
2% agarose gel	1 g agarose 50 ml 0.5X TBE
0.5 M EDTA	186.12 g EDTA adjusted to 1 L with dH_2O
LB medium	10 g Bacto-Tryptone 5 g Bacto-yeast 10 g NaCl adjusted to 1 L with dH_2O
LB plates	10 g Bacto-Tryptone 5 g Bacto-yeast 10 g NaCl 15 g Bacto-agar adjusted to 1 L with dH_2O
Lysis buffer	0.11 M $NaCl_2$ 55 mM Tris pH 8.0 1.1mM EDTA pH 8.0,

	0.55% sodium dodecyl sulphate [SDS] 1 mg/ml Proteinase K 40 μg/ml poly A adjusted to 1 L with dH_2O
PBS	8 g NaCl 0.2 g KCl 1.44 g Na_2HPO_4 0.24 g KH_2PO_4 adjusted to 1 L with dH_2O
Sanger TBE	324 g Tris base 85 g boric acid 19 g EDTA adjusted to 2 L with dH_2O
3 M sodium acetate (NaAc)	40.8 g NaAc adjusted to 100 ml with dH_2O
0.9% sodium chloride (NaCl)	0.9 g NaCl adjusted to 100 ml with dH_2O
10% SDS	100g SDS adjusted to 1 L with dH_2O
10X SSC	175.32 g sodium chloride 88.23 g sodium citrate adjusted to 1 L with dH_2O
SOC medium	5 g Tryptone Peptone

	1.25 g yeast extract
	0.15 g NaCl
	0.05 g KCl
	0.51 g $MgCl_2$
	2.5 ml $MgSO_4$
	10 ml 20% glucose
	adjusted to 1 L with d H_2O
0.5X TBE	54 g Tris base
	27.5 g boric acid
	20ml 0.5 M EDTA
	adjusted to 1 L with dH_2O

Table 2.2.1 – Table detailing the components of solutions used in this thesis.

2.3 Samples

Minipool plasma samples were acquired from the Scottish National Blood Transfusion service which assures the safety of all blood donated in Scotland and Northern Ireland by testing for HCV and HIV by PCR. The sensitivity of this assay allows for this testing to be carried out on 95 individual donations amalgamated into a single 'minipool' sample. The minipools used in this study were all HIV, HCV and HBV PCR negative. Fifteen Taiwanese minipools were also used to confirm the sensitivity of the SENV assay developed for this project.

One hundred and ninety two human plasma samples were obtained from the Scottish National Blood Transfusion Service. These had been sent to the PCR Reference Laboratory for confirmatory testing following indeterminate serology results. All of the samples had been HIV, HCV and HBV negative by PCR testing.

Six post mortem, HIV positive, bone marrow samples and nineteen post mortem, HIV positive, paired, bone marrow and spleen samples were supplied by the Medical Research Council Brain and Tissue Bank of Edinburgh (Western General Hospital, Edinburgh). Bone marrow, spleen, left cerebellum, lymph node, thoracic spinal cord and liver from the

same HIV positive individual were also provided by the same source. Seven post mortem, HIV negative, paired, bone marrow and spleen samples were supplied from Medical Research Council Sudden Death Brain & Tissue Bank and the same number of bone marrow aspirate samples were collected from patients investigated for haematological abnormalities at the Royal Infirmary, Edinburgh. The use of tissue samples was approved for research by the Lothian Ethics of Research committee.

Ten serum samples from blood donors with either HIV, HCV or HBV and 10 samples from HIV/HCV/HBV negative donors were supplied by the Scottish National Blood Transfusion Service.

Twenty serum samples from patients undertaking an immunosuppressive drug regime following heart, lung or both heart and lung transplant surgery were collected previously for a study investigating Epstein-Barr virus and were used with the permission of Professor Dorothy Crawford and Dr Paul Hopwood.

Sera from 13 drills, two mandrills, four cherry-capped mangabeys, five chimpanzees, 14 orangutans, 19 gibbons and one gorilla were selected as representative samples from non-human primates and investigated for the presence of TTV/TLMV. These were collected by sanctuaries in Nigeria, Cameroon and Taiwan with the exception of the gorilla serum, which was provided by Edinburgh Zoo. Again with the exception of the gorilla, all of the primates had been wild caught and each of the species were housed separately from other primate species.

The Roslin Institute, Edinburgh, made sera from 20 cows, 20 sheep, 20 goats and 29 chickens available. These samples had been collected from animals kept in a variety of locations in England and Scotland.

2.4 Nucleic Acid extraction

2.4.1 Nuclisens Method

The nuclisens extraction method (bioMérieux) is a guanidine thiocyanate and silica based semi automated extraction method in which between 200 µl and 1 ml of the samples was added to a lysis buffer containing 5 mol/l guanidine thiocyanate, Triton X-100 and Tris/HCl which had been pre-incubated at 37°C. Fifty µl of hydrochloric acid-activated silicon dioxide particles was then added to each sample and these were incubated for 30 minutes at 37°C, after which the samples were centrifuged for two minutes at 13000 rpm

to produce a silica pellet. The pellet was resuspended in a small volume of the lysis buffer and added to the extractor cartridge (Figure 2.4.1). Air pressure applied into the cartridge resulted in the sample being forced through the cartridge leaving the silica particles binding to the filter. Multiple wash cycles are performed with a wash buffer containing guanidinium thiocyanate as well as with acetone and 70% ethanol. The silica was air dried at 56°C before the nucleic acid was eluted in elution buffer (Tris/HCl) at 56°C and transferred to a clean tube at the bottom of the cartridge.

2.4.2 Phenol-Chloroform Method

One hundred µl of serum or plasma was added to 400 µl of lysis buffer which had been pre-incubated at 37°C to inactivate endogenous RNAses. This was then incubated for a further 10 minutes at 37°C. A portion of tissue equivalent to 0.5-1.0 cm^3 was dissected from frozen tissue samples and added to 500 µl of pre-incubated lysis buffer. These were then incubated overnight at 56°C and vortexed frequently to aid the digestion process. Once the serum, plasma and tissue samples had been lysed, extraction was carried out with the addition of 450 µl of water-saturated phenol (Sigma) to separate the protein from the nucleic acid.

After vortexing for 5 minutes, the samples were centrifuged for 10 minutes at 13000 g. The aqueous phase was then removed and added to an eppendorf containing 450 µl 50:1 chloroform:iso-amyl alcohol (BDH) solution. This was then vortimixed for a further 2 minutes before being centrifuged for 5 minutes at 13000 g. The aqueous phase was removed and transferred to an eppendorf to which 40 µl 3 M Sodium Acetate pH 5.2 had already been added. One ml of 100% ethanol (-20°C) was then added and the tubes inverted to mix the solution, which was then left overnight at -20°C to allow the nucleic acids to precipitate. The nucleic acids were pelleted by centrifugation at 13000 g for 10 minutes (0°C) and washed with 600 µl 80% (v/v) ethanol. The resultant pellet was left to air dry at 37°C for 10 minutes to allow any remaining ethanol to evaporate before being resuspended in 20 µl RNase/DNase free water (Promega).

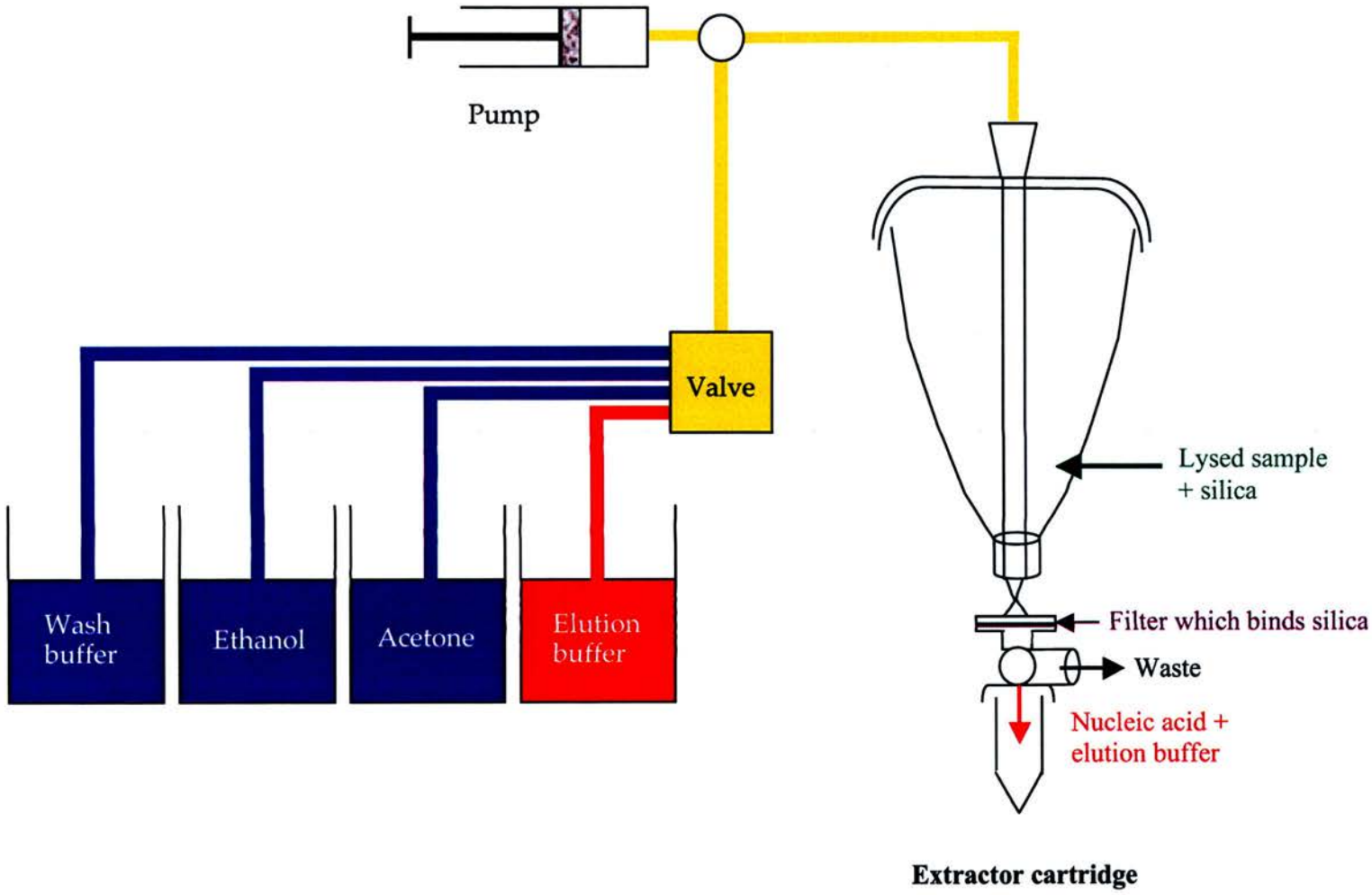

Figure 2.4.1 – Schematic diagram of nucleic acid extraction using a Nuclisens automated extractor.

2.4.3 High Pure PCR Template Preparation kit

Nucleic acids were extracted from serum and tissues using the High Pure PCR Template preparation kit (Roche) following the manufacturers instructions. Briefly, 25-50 mg tissue was incubated at 55°C for 1 hour with tissue lysis buffer (4 M urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA) and proteinase K. Following the addition of binding buffer (6 M guanidine HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100), the sample was incubated a 70°C for 10 minutes. Serum (200 µl) was added to binding buffer and proteinase K and also incubated at 70°C for 10 minutes. The remaining extraction method was the same for both tissue and serum and involved the addition of isopropanol before the solution was centrifuged through a glass fibre packed filter tube to capture the nucleic acids. Contaminating cellular components were removed during a series of washing steps with inhibitor removal buffer (5 M guanidine-HCl, 20 mM Tris-HCl) and wash buffer (20 mM NaCl, 2 mM Tris-HCl). Finally, a low salt buffer (10 mM Tris) was used to elute the nucleic acids from the glass fibre.

2.4.4 QIAamp MinElute Virus Spin kit

Nucleic acids from serum samples were extracted using QIAamp MinElute Virus Spin kit (Qiagen) following the manufacturers protocol. If a serum sample volume was less than 200 µl, it was first made up to the appropriate volume using 0.9% sodium chloride solution then protease and buffer AL added. This was incubated at 56°C for 15 minutes and 100% ethanol added before being transferred to a column. The sample was centrifuged to remove the lysate and the nucleic acid bound to the filter washed with buffer AW2 and 100% ethanol. The nucleic acid was eluted from the membrane using buffer AVE.

2.5 Quantitation of Nucleic Acids

2.5.1 Quantitation by spectrophotometry

The extracted nucleic acids were analysed on a spectrophotometer to determine the concentration of the DNA and the purity of the sample. Spectrophotometric absorbance reading were taken at 260 nm, the wavelength at which DNA absorbs light, and 280 nm, which is the absorbance maxima of proteins.

The concentration of DNA calculated using the equation:

DNA concentration (µg/ml) = A_{260} X D X 50

Where A_{260} is the optical density of the sample at 260 nm, D is the dilution factor and 50 (μg/ml) is the concentration of double stranded DNA that results in an A_{260} of 1.

The A_{280} gave an estimate of the concentration of protein within the samples. The purity of the sample was ascertained using the ratio of A_{260}/A_{280} where a value of >1.5 was deemed to be sufficiently pure.

2.5.2 Quantitation by limiting dilution

The sensitivity of the PCR reaction and its ability to amplify single molecules of target DNA allows for the quantification of viral genomes by limiting dilution. This involved the titration of a DNA samples in ten fold serial dilution steps until the endpoint is achieved. The last PCR positive dilution is considered to contain at least one copy of the molecule of interest. To quantify the titre of TTV and TLMV, the samples were subject to 10 fold serial dilution before being amplified using UTR nested PCR or TTV/TLMV specific PCR methods (see section 2.6).

The titre of TTV/TLMV was estimated using the following calculation:

Total DNA in one cell = 6.6 pg ≡ 6.6×10^{-6} μg ≡ 10^{-6} cells containing 6.6 μg DNA

DNA concentration of sample = Y μg/μl

Number of cells per μl (n) = Y/6.6×10^{-6} μg

Number of cells added to 1st dilution of dilution series (N) = n X number of μl neat DNA added to 1st dilution of dilution series

Number of cells added to primary PCR (Z)= (no. of μl of 1st dilution added to 1° PCR / total volume of 1st dilution in dilution series) X N

Dilution 1 contains Z cells
Dilution 2 contains Z/10 cells
Dilution 3 contains Z/100 cells.....etc

Approximate number of viral copies per cell = 1 /number of cells in last positive dilution.

2.6 Polymerase Chain Reaction

PCR allows the amplification of a target region of DNA from a sample containing heterogeneous DNA sequences. Heating of the sample forces the DNA to denature and allows two small oligonucleotides, which are synthesised to complement the sequence of regions flanking the target DNA, to hybridise to opposite strands of the now single stranded DNA. A thermostable DNA polymerase then catalyses the extension of the primer sequence in a 5' to 3' direction. Multiple cycles of this denaturation, primer annealing and extension results in the almost exponential amplification of the target DNA sequence. Nested PCR, in which a second set of primers internal to the first set are used in a secondary reaction, increases the sensitivity and specificity of the reaction allowing even single molecules in a heterogeneous DNA sample to be detected.

All PCR reactions used *Taq* DNA polymerase in storage buffer B: 20 mM Tris-HCl pH 8, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20 and 0.5% Nonidet-P40 and *Taq* 10X buffer: 500 mM KCl, 100 mM Tris-HCl pH 9, 15 mM $MgCl_2$ and 1% Triton X100 (Promega). Sequences of the primers used in the following reactions are shown in Table 2.6.1. All PCR reactions contained nucleic acid extracted from minipool plasma as positive controls and water as negative controls unless otherwise stated. Frequently a large number of PCR reactions were carried out simultaneously with control reactions and visualised on either one large agarose gel or several smaller ones. For this reason, it has not always been possible to include control reactions in the figures presented in this thesis.

Primer set	Primer name	Primer: Inner/Outer (I/O), Sense/Anti-sense (S/AS)	Sequence 5'→3'	Reference
UTR primers	CVOA	O/AS	AGCCCGAATTGCCCCTWGACT	
	CVOS	O/S	TRCACWKMCGAATGGCTGAGTTT	
	CVIA	I/AS	CTCACCTYCGGCWCCCGCCC	
N22 primers	NG059	I/S	ACAGACAGAGGAGAAGGCAACATG	[Okamoto, 1998]
	NGO61	O/S	GGCAACATGTTATGGATAGACTGG	[Okamoto, 1998]
	NG063	O/AS	CTGGCATTTTACCATTTCCAAAGTT	[Okamoto, 1998]
N22/2 primers	F1	O/S	GTGGGACTTTCACTTGTCGGTGTC	[Leary et al., 1999]
	R1	O/AS	GACAAATGGCAAGAAGATAAAGGC C	[Leary et al., 1999]
	F2	I/S	AGGTCACTAAGCACTCCGAGCG	[Leary et al., 1999]
	R2	I/AS	GCGAAGTCTGGCCCCACTCAC	[Leary et al., 1999]
TTV specific primer	TTV	O/S	AACMKGGTCTACRTCCTSATATAA	
TLMV specific	TLMV	O/S	GGAGGAGHHWMHMHACTATATAA	

Table 2.6.1 – TTV/TLMV PCR primer sequences.

2.6.1 UTR primers

The UTR primers are situated in the partially conserved untranslated region of the genome and ambiguities were introduced into the primer sequences to allow both TTV and TLMV to be amplified simultaneously (Figure 2.6.1).

In the primary PCR reaction, 5 µl of DNA was added to a total volume of 50 µl containing 5 µl 10X PCR buffer, 30 µM of dATP, dTTP, dCTP and dGTP, one unit *Taq* DNA polymerase and 0.25 µM of primers CVOS and CVOA. A secondary reaction was carried out by transferring 2 µl of the primary reaction to a final reaction volume of 20 µl in which the primers CVOS and CVIA were used. Both the primary and secondary PCRs were amplified using 30 cycles of 94°C for 18 seconds, 55°C for 21 seconds and 72°C for 90 seconds.

2.6.2 N22 primers

The N22 primers are situated in the coding region of the TTV genome and were used in some of the original epidemiological studies (Okamoto et. al. 1998). The reaction mix and amplification program were the same as for the UTR PCR but primers NG059 and NG063 were used in the primary reaction and NG059 was substituted for NG061 for the secondary PCR.

2.6.3 N22/2 primers

The N22/2 primers are a fully nested primer set which amplifies a region of the TTV coding sequence. The primary PCR used 2 µl of total nucleic acids in a final volume of 10 µl which included 1 µl of 10X PCR buffer, 1 µM of primers F1 and R1, 2 µM $MgCl_2$ and 0.75 units of *Taq* polymerase. In the nested PCR reaction, 1 µl of the primary product was transferred to a final volume of 25 µl which contained 2.5 µl 10X PCR buffer, 0.5 µM of primers F2 and R2, 2mM MgCl2 and 0.625 units of Taq polymerase. Amplifications for both the primary and secondary PCRs were 35 cycles: 20 seconds at 94 °C, 30 seconds at 55 °C and 30 seconds at 72°C.

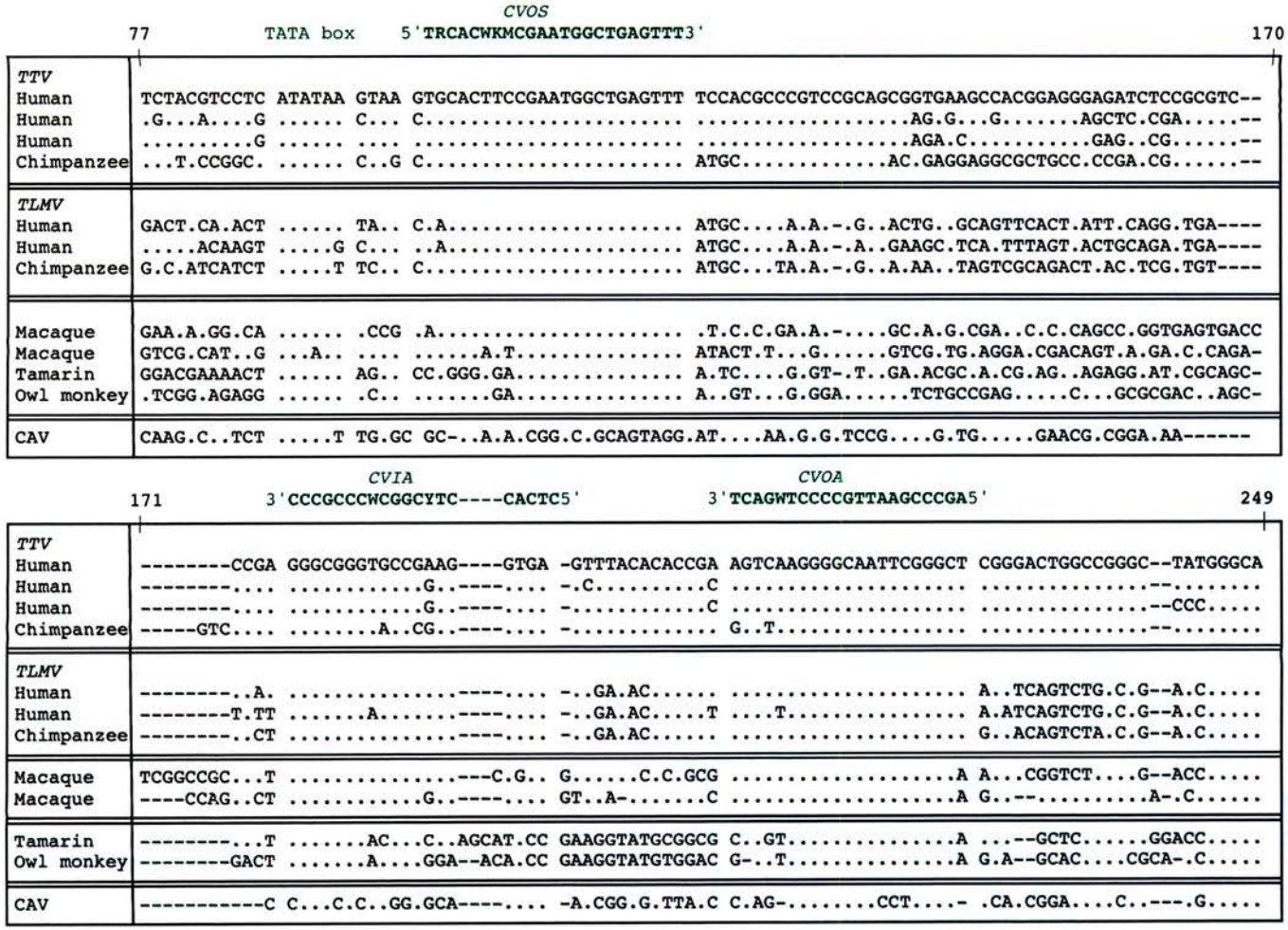

Figure 2.6.1 - Alignment of the UTR region of TTV and TLMV from humans and non-human primates, showing the position of the primers CVOS, CVOA and CVIA. Also included is the same region from Chicken Anaemia Virus (CAV)

2.6.4 TTV/TLMV specific primers.

In order to amplify only TTV or TLMV from a sample, primers specific for both viruses were designed upstream of the UTR primer CVOS. A fully nested PCR was carried out with the TTV or TLMV specific primer and CVOA utilised in the primary reaction and CVIA and CVOS in the secondary reaction. The reaction mix and conditions were the same as for the UTR primers.

2.7 Agarose gel electrophoresis

Gel electrophoresis allows PCR products to be separated because they migrate at different rates when a current is applied across the gel. Products from the secondary PCR reaction (20 µl) were run on 2% agarose gels (Sigma) in a flatbed electrophoresis rig. A 0.5X TBE solution was used both in the making of the gel and as the electrophoresis buffer. The gels were stained with 0.1 µg/ml ethidium bromide which binds to the DNA and allows it to be visualised under ultraviolet (UV) light. All gels were run at 150 volts for between 25 minutes and 1 hour with an appropriately sized marker to accurately determine the size of the products. Unfortunately, due to the number of PCR products run simultaneously on the agarose gels, it has not always been possible to include the size marker in all of the figures included in this thesis

2.8 Lightcycler Real-Time PCR

The Roche Lightcycler enables 'real-time PCR' making it possible to visualise the accumulation of the PCR products during amplification. This is because unlike other themalcyclers, which use solid thermal blocks, the Lightcycler uses air as the medium of heat conduction. The reactions take place in thin walled glass capillaries that have a high surface to volume ratio, allowing very efficient heat transfer. There are two detection formats, which can be used with the Lightcycler; hybridisation probes which are based on FRET (Fluorescence Resonance Energy Transfer) technology, and SYBR green.

Hybridisation probes are sequence specific oligonucleotides labelled with fluorescent dyes which bind within 1-5 base pairs of each other, internal to the PCR primers, during the annealing stage of amplification. On excitation by an LED the 'donor' probe emits energy which in turn excites the acceptor probe. This then fluoresces at a different wavelength from the original LED and the fluorescence is detected and quantified by the Lightcycler (Figure 2.8.1). The accumulation of the target PCR product as cycling progresses allows more probes to bind, which in turn causes a detectable increase in fluorescence.

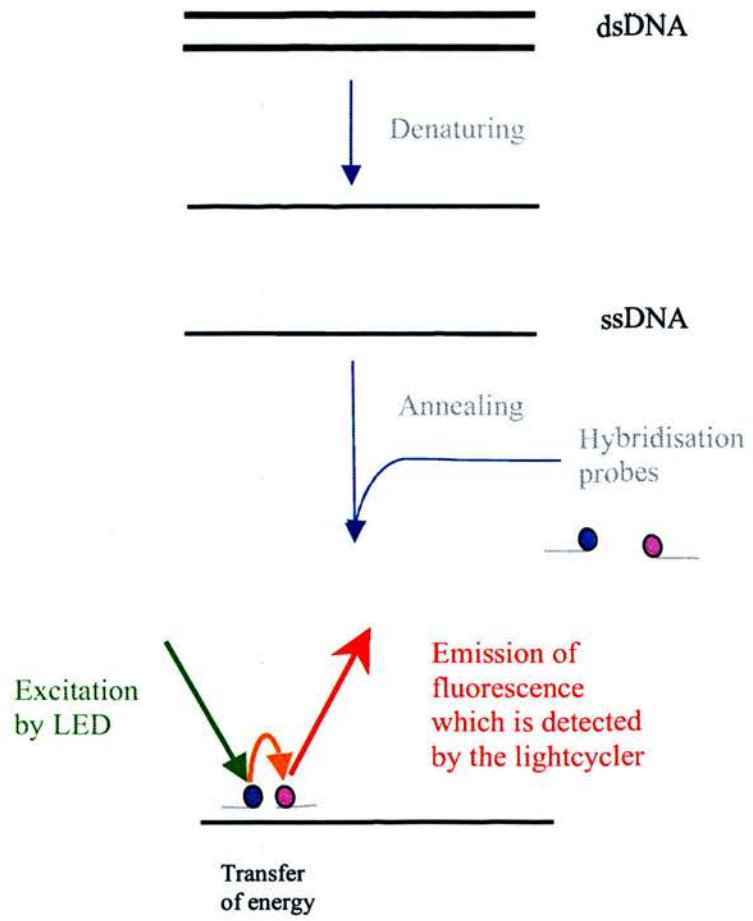

Figure 2.8.1 – Real-time PCR using hybridisation probes.

The second format uses SYBR green, a fluorescent dye which binds to the minor groove of double stranded DNA as is synthesised by the polymerase during the elongation stage of cycling, and emits light when excited by an LED (Figure 2.8.2). Again, the accumulation of the target DNA sequence as the reaction proceeds results in an increase in dsDNA available for the SYBR green and therefore an increase in the fluorescence.

2.8.1 PCR reaction using SYBR green format

Two µl of the primary PCR reaction using the UTR primers was added to a final reaction volume of 20 µl containing 2 µl Lightcycler FastStart reaction mix SYBR Green 1 (FastStart Taq DNA polymerase, reaction buffer, dNTP mix, SYBR green 1 dye and 1 mM $MgCl_2$) (Roche), 3 mM $MgCl_2$ and 1 µM of UTR secondary PCR primers CVOS and CVIA. The reaction was carried out using the following conditions: 95°C for 2 seconds, 55°C for 5 seconds and 72°C for 7 seconds.

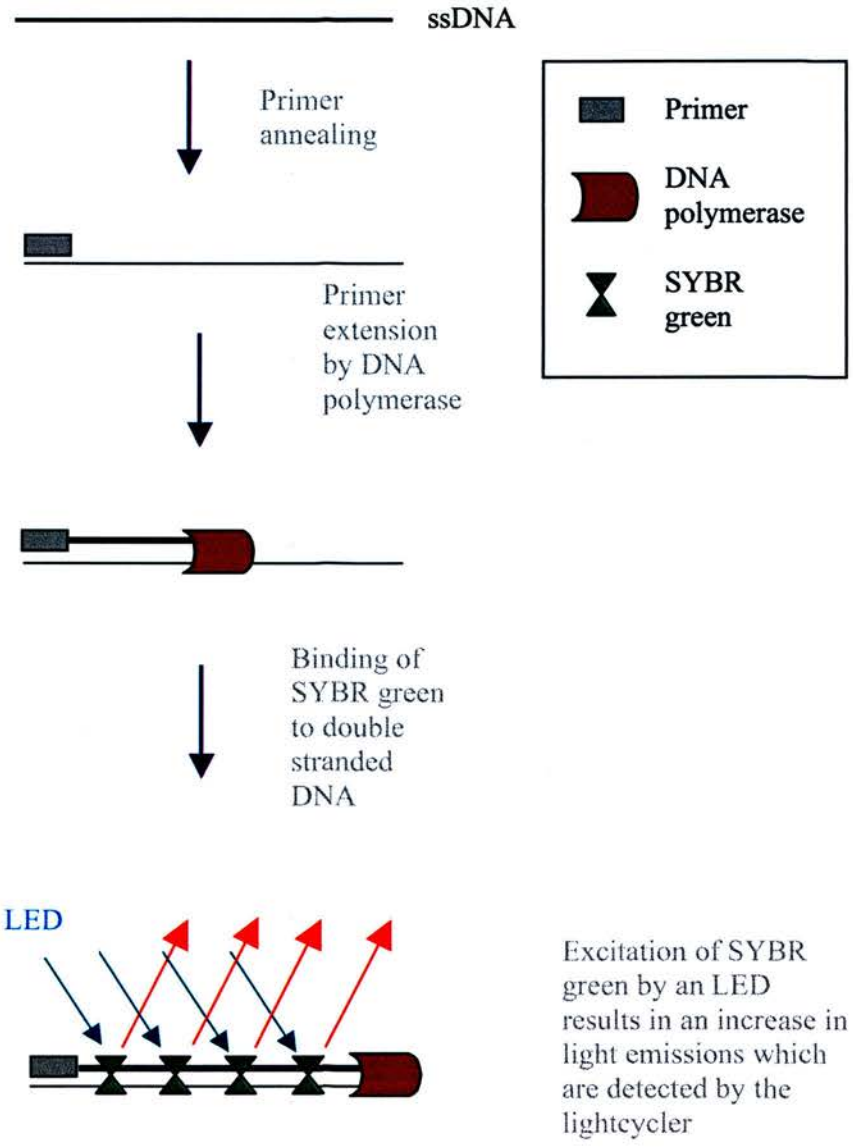

Figure 2.8.2 – Real-time PCR using SYBR green.

For melting curve analysis to take place, following amplification the PCR products were heated to 95°C to allow complete denaturation of the products. Cooling to 60°C ensured the products were double stranded before being heated to 95°C in 0.1°C/second intervals to determine the exact temperature each of the products dissociated from the complementary strand and became single stranded.

2.9 Hepatitis C Virus PCR

A nested HCV PCR reaction was carried out using primers situated in the 5' non- coding region (5'NCR) and part of the core region. The first round was carried out using a one tube, two enzyme Access PCR system (Promega) to allow the generation of cDNA from the RNA, followed by amplification of the cDNA template. Five µl of nucleic acid was added to a reaction mixture which contained 1X AMV/*Tfl* reaction buffer, 0.2 µM of dNTP mix (containing 10 mM of each dNTP), 1 µM of primers Z4130 and Z4133 (Table 2.9.1), 1 mM Mg_2SO_4, 0.1 unit/µl AMV reverse transcriptase, 0.1 unit/µl of *Tfl* DNA polymerase and nuclease free water to a final volume of 50 µl. Conditions for this reaction were 48°C for 45 minutes for reverse transcription of RNA, 2 minutes at 94°C to inactivate the reverse transcriptase and denature the RNA/cDNA and primers. This was followed by 40 cycles of 94°C for 30 seconds, 55°C for 21 seconds and 72°C for 1.5 minutes.

Two µl of the primary PCR reaction were transferred into a secondary PCR carried out on the Roche Lightcycler using the hybridisation probe method outlined in Figure 2.8.1. A final volume of 20 µl containing 4 mM of $MgCl_2$, 0.4 µM of the probes HCV-P and HCV-F (Table 2.9.2), 0.5 µM of primers Z4131 and Z4132 (Table 2.9.1) and 1X DNA Master Hybridisation Probes (Roche). Amplification is carried out using the following cycling parameters: 94°C for 2 minutes followed by 25 cycles of 94°C for 2 seconds, 60°C for 10 seconds and 72°C for 25 seconds

Primer set	Primer name	Primer: Inner/Outer (I/O), Sense/Anti-sense (S/AS)	Sequence 5'→3'	Reference
HCV primers	Z4130	O/S	CGGGAGAGCCATAGTGGTCTG	[Cleland, 1999]
	Z4133	O/AS	GGTTTAGGAWKYGTGCTCATG	{Cleland, 1999 #583
	Z4131	I/S	GAACCGGTGAGTACACCGGAA	{Cleland, 1999 #583
	Z4132	1/AS	TGCACGGTCTACGAGACCTCC	{Cleland, 1999 #583

Table 2.9.1 – Primer sequences for HCV PCR.

Probe name	Fluorophore	Sequence 5'→3'	Reference
HCV-F	3'-flourescein	CGAAAGGCCTTGTGGTACTG	[Jarvis, 2005]
HCV-P	5'-LC Red 705	CTGATAGGGTGCTTGCGAGT	[Jarvis, 2005]

Table 2.9.2 – Hybridisation probes used for the detection of HCV.

2.10 Molecular cloning

PCR products were cloned using pGEM-T Easy Vector System and JM109 competent cells (Promega). This system incorporates a 3' thymidine overhang into the vector that increases the efficiency of the ligations and is compatible with the 3' deoxyadenosine generated by the *Taq* polymerase used to generate the PCR products. The multiple cloning site for this vector is situated within the coding region for the enzyme β-galactosidase, allowing identification of recombinant clones by blue/white colour screening.

2.10.1 Ligations

The PCR product concentration was determined by comparison to a sample of known concentration when both were run simultaneously on an agarose gel. The amount of PCR product required for optimal insert:vector ratios was then calculated using the following equation.

$$\frac{\text{ng of vector (50 ng)} \times \text{Size of insert (kb)}}{\text{Size of vector (3.0 kb)}} \times \text{Insert:vector molar ratio} = \text{ng of vector}$$

Table 2.10.1 shows the ligation reaction which was set up and left overnight at 4°C to help produce the maximum number of transformants.

2.10.2 Transformations

The eppendorfs containing the ligation reactions were centrifuged and 2 µl was transferred to a 1.5 ml microfuge tube which had already been placed on ice. JM109 high efficiency competent cells (genotype *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (r_{k-}, m_{k+}), *rel*A1, *sup*E44, (*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*Z M15]) were thawed on ice and mixed gently before 50 µl was added to each of ligation reactions.

Reagent	Volume
2X rapid ligation buffer, T4 DNA ligase (60 mM Tris-HCl, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol)	5 µl
pGEM-T easy vector (50 ng)	1 µl
PCR product	X µl
T4 DNA ligase (3 Weiss units/µl)	1 µl
Deionised water	10-X µl

Table 2.10.1 – Ligation reaction for pGEM-T easy vector system.

After 20 minutes incubation on ice the cells were 'heat-shocked' by being placed in a 42°C water bath for 45-50 seconds before being returned to the ice. Nine hundred µl of SOC medium was added to each tube and the reactions were left for 1.5 hours at 37°C in a shaking incubator. After transformation, 100 µl of each culture was plated on LB agar plates supplemented with 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. The plates were incubated for between 16 and 24 hours at 37°C.

2.10.3 Screening colonies

In recombinant plasmids the insert disrupts the LacZ coding region, resulting in white bacterial colonies which can easily be differentiated from cells with the non-recombinant plasmid, which are coloured blue. Cells from white colonies were screened by using a toothpick to transfer a small number of cells to a PCR reaction that contained primers CVIA and CVOS (Table 2.6.1). The PCR products were visualised either on an agarose gel (section 2.7) or by using the Lightcycler (2.8.1).

2.10.4 Plasmid DNA minipreps

LB media was supplemented with 50 µg/ml of ampicillin and 3 ml was aliquoted into 15 ml sterile tubes. These were seeded with the colonies which had already been PCR screened to confirm the presence of the insert and were left overnight at 37°C in the shaking incubator to produce bacterial cultures. 1.5 ml of the resulting culture was

transferred into a 1.5 ml eppendorf tube and the DNA extracted using QIAprep spin miniprep kit (Qiagen). Cells were harvested by centrifugation for 5 minutes at 6500 rpm and the supernatant removed. The cells pellet was resuspended in 250 µl of resuspension buffer P1 and transferred to a microfuge tube before the addition of 250 µl of lysis buffer P2. This solution was inverted gently until it became viscous and slightly transparent after which 350 µl of neutralisation buffer N3 was added and the solution mixed by inversion again. A compact white pellet was formed after centrifugation for 10 minutes and the supernatant decanted to a QIAprep spin column. The spin column was centrifuged for 1 minute to allow the DNA to bind to a membrane and the flow through discarded. The bound DNA was washed with first with 0.5 ml of buffer PB then with 0.75 ml buffer PE, both of these buffers were removed in turn by centrifugation for 1 minute. Finally, 50 µl of water was added onto the centre of the spin column and allowed to stand for 1 minute before being centrifuged for 1 minute to elute the DNA.

2.10.5 Restriction digests

The multiple cloning site of pGEM-T Easy vector is flanked by restriction enzyme sites which facilitate the removal of the insert from the vector using a single digestion (Figure 2.10.1).

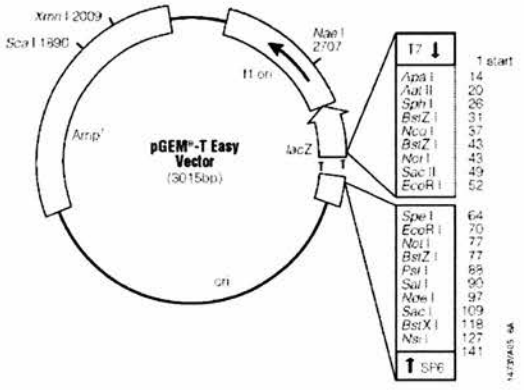

Figure 2.10.1 - Restriction map of pGEM-T Easy vector.

The restriction enzyme *Eco* RI (Promega) was used to cut the vector at two sites, and the digested DNA analysed to ensure the clones contained the desired inserts. The recognition sequence for *Eco* RI is shown below (Figure 2.10.2). Two µl of DNA (see section 2.10.3) was added to a reaction mix containing 1 µl 10X buffer H (90 mM Tris-HCl, 10 mM

$MgCl_2$, 50 mM NaCl), 1 µl *Eco RI*, 0.1 µl BSA and 5.9 µl dH_20 and incubated overnight at 37°C.

Figure 2.10.2 - Recognition sequence for restriction enzyme *Eco* RI.

All 10 µl from the overnight reaction was run on a 2% agarose gel (see section 2.7) and any bands visible on the gel compared with a DNA marker of a known size to confirm it corresponds to the insert excised from the plasmid.

2.11 Sequencing of cloned DNA fragments

Manual DNA sequencing was performed using a thermosequenase radiolabelled terminator cycle sequencing kit (Amersham). This is based on the Sanger sequencing method which, like PCR, involves the amplification of a DNA sequence using sequence specific oligonucleotides complementary to the DNA strand of interest to 'prime' the start of replication. DNA polymerase catalyses the reaction and deoxynucleotides are incorporated as the 'building blocks' for the new strand. The sequence is determined using DNA chain terminating [α-33P] labelled di-deoxynucleoside triphosphates (ddNTPs)(PerkinElmer), which are integrated into the DNA, resulting in truncated DNA terminated only at a position where a particular ddNTP has been incorporated. The fragments can be separated according to size by electrophoresis on a polyacrylamide gel. Using this method, when reactions for all four of the radiolabelled bases (ddGTP, ddATP, ddTTP and ddCTP) are run side by side (as shown in Figure 2.11.1), the original DNA sequence can be determined.

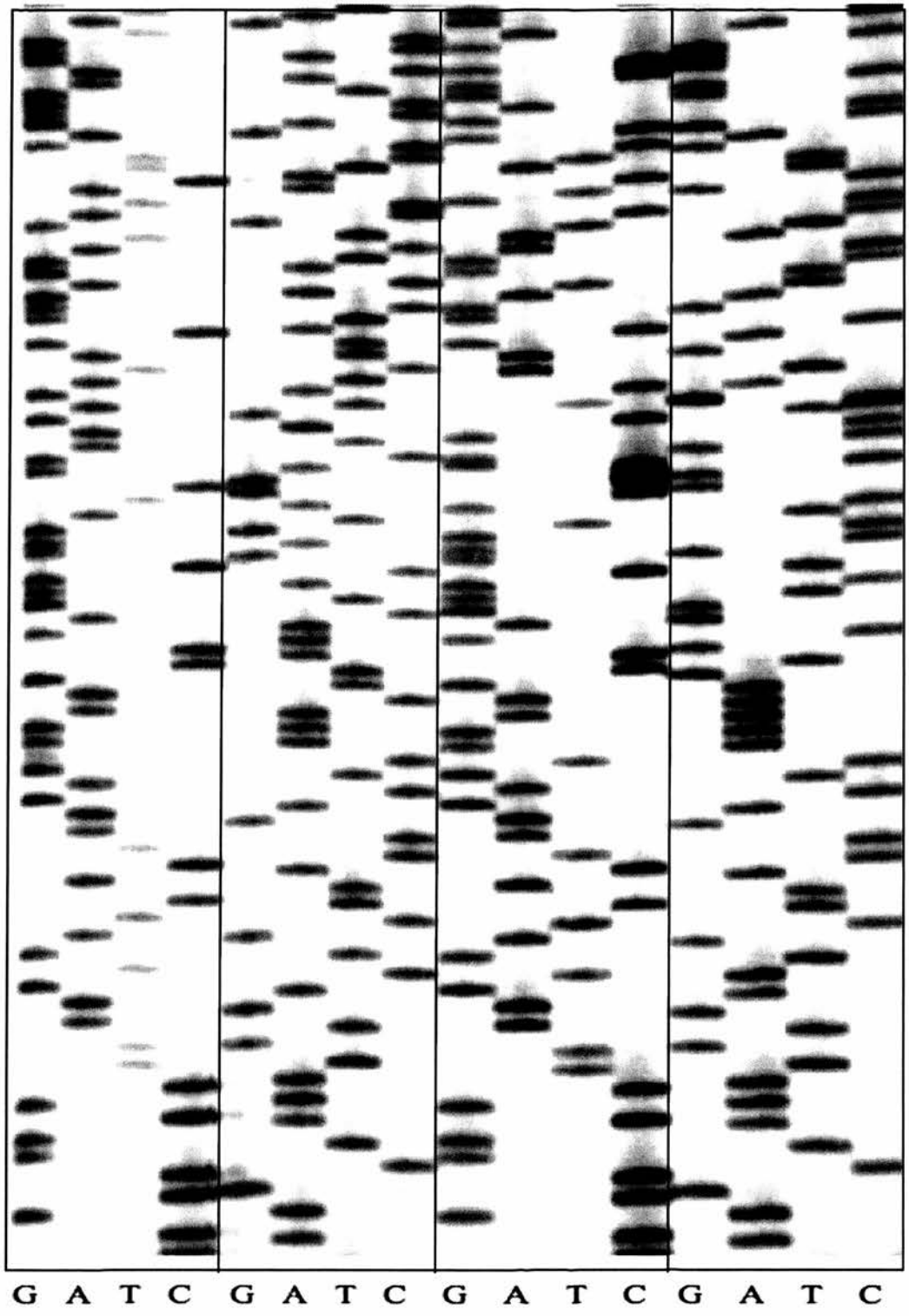

Figure 2.11.1 – Representative film from cycle sequencing reaction.

Following DNA recovery using QIAprep spin miniprep kit (section 2.10.4), 50-500 ng of DNA was added to a sequencing reaction which contained 2 µl reaction buffer (260 mM Tris-HCl (pH 9.5), 65 mM $MgCl_2$), 10 pmoles M13 forward or reverse primer, 0.5 µl thermosequenase enzyme (4 units/µl thermosequenase DNA polymerase enzyme, 50 mM Tris HCl (pH 8), 1 mM DTT, 0.1 mM EDTA, 0.5% Tween-20, 0.5% nonidet P-40, 50% glycerol) and water to a final volume of 20 µl.

Termination mixes were made by adding 2 µl of dGTP termination master mix (7.5 µM dGTP, 7.5 µM dATP, 7.5 µM dTTP and 7.5 µM dCTP) with 0.5 µl of α-^{33}P labelled ddNTP, one for each base. Four tubes were labelled (G, A, T and C), and 2.5 µl of termination mix and 5 µl of the DNA/reaction buffer mix added to each tube. The reactions were overlayed with mineral oil and subjected to 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for one minute. Following the thermal cycling, 6 µl of each reaction was transferred into the well of a 96 well microtitre plate containing 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF).

2.11.1 Polyacrylamide gel electrophoresis

The sequencing products were resolved by electrophoresis through a 6% polyacrylamide gel. Glass plates were cleaned thoroughly with distilled water, methanol and finally acetone and placed together, separated by 1 mm spacers. The gel mixture was prepared by dissolving 21 g urea (BDH), 6 ml Sequagel XR concentrate (National Diagnostics), 5 ml 10X Sanger TBE and 0.05 g ammonium persulphate (APS)(Sigma) in distilled water to a final volume of 50 ml. After polymerisation of the gel was initiated by the addition of 20 µl TEMED (N,N,N',N,-tetramethyethyediamine)(Sigma), the gel immediately poured slowly between the plates to avoid the incorporation of bubbles and left to set for 1 hour. Denatured samples were loaded into the wells of the gel in the order G, A, T and C and the samples electrophoresed at 75 watts for approximately 2.5 hours or until the bromophenol blue was visible at the bottom of the gel using 1X Sanger TBE as the electrophoresis buffer. The gel was removed from the glass plates and transferred onto filter paper before being vacuum dried at 80°C for 1.5 hours and exposed to X-ray film (Kodak) for 1-2 days depending on the intensity of the signal. Sequencing gels were read and manually aligned using Simmonic 2000 software package developed by Professor Peter Simmonds.

2.12 Southern blotting

Southern blotting is a method that allows the detection of sequences, which have been transferred from an agarose gel to a membrane, by hybridising them with a sequence specific labelled probe and visualising them using an appropriate detection system.

2.12.1 Amplification and gel electrophoresis

Initially a single round of amplification was carried out with either primer D10S (SENV D) or C5S (SENV H) with L2AS (Table 2.12.1). Ten µl of nucleic acid was added to 40 µl of mastermix containing one unit of *Taq* polymerase, 30 µM of dATP, dTTP, dCTP and dGTP, and 0.25 µM of each of the primers. Amplification was carried out at 94°C for 10 minutes followed by 45 cycles of 94°C for 25 seconds, 58°C for 60 seconds and 72°C for 30 seconds. The products were electrophoresed on a 1.2% agarose gel at 150 V for 30 minutes.

To increase the sensitivity of the PCR, outer primers (SENV sense and SENV anti-sense (Table 2.12.1)) were designed to amplify both SENV D and SENV H simultaneously. These were used as a primary PCR reaction followed by a secondary PCR using the primers and conditions described above. Amplification for the primary PCR was carried out using 30 cycles of 94°C for 18 seconds, 55°C for 21 seconds and 72°C for 90 seconds.

Ten µl of the secondary product was mixed with 3 µl 6X loading dye and wet loaded into alternate wells of a 1.2% agarose gel stained with 25 µg EtBr. Three µl of a digoxigenin labelled DNA molecular weight marker was also run on the gel to allow comparison of the fragment sizes. The gel was run at 110 V for 45 minutes in a flat bed gel tank containing 0.5X TBE buffer supplemented with 250 µg EtBr after which the gel was visualised under UV light and a picture taken for reference purposes. The gel was then washed in 0.25 N HCl solution for 10 minutes to allow depurination before the DNA in the gel was denatured by washing in a 0.5 N NaOH and 1.5 M NaCl solution for 30 minutes. The denaturation solution was neutralised using a 0.5 M Tris and 1.5 M NaCl solution in which the gel was left for 30 minutes. All the washes were carried out on an agitator.

Primer set	Primer name	Primer: Inner/Outer (I/O), Sense/Anti-sense (S/AS)	Sequence 5'→3'	Reference
SENV primers	SENV sense	O/S	TTGAAGACMASTGGYACASYCAGC	Thom (unpublished)
	SENV anti-sense	O/AS	CCTATRAAWATRTTTGARTACCAGCC	Thom (unpublished)
	D10S (SENV D)	I/S	GTAACTTTGCGGTCAACTGCC	[Umemura et al., 2001a]
	C5S (SENV H)	I/S	GGTGCCCCTWGTYAGTTGGCGGTT	[Umemura et al., 2001a]
	L2AS	I/AS	CCTCGGTTKSAAAKGTYTGATAGT	[Umemura et al., 2001a]

Table 2.12.1 - SENV D and H PCR primer sequences.

2.12.2 Transfer of DNA to nylon membrane

Two pieces of 3 mm Whatman paper (Camlab), a piece of sponge and a sheet of positively charged nylon membrane (Roche) were cut to size 1 cm larger than the size of the gel and soaked in 10X SSC buffer. The Whatman paper, membrane, gel and sponge were placed on the porous membrane support pad of a Posiblot 30-30 pressure blotter in the order shown in Figure 2.12.1. To ensure the complete saturation of the sponge with transfer buffer, 10X SSC was pored onto the sponge until it began to seep out of the bottom edges. The posiblot unit was closed and sealed and a hose connected to a pressure control station attached. The pressure was adjusted to 75 mm mercury (Hg) and the system left for 1 hour to allow the complete transfer of the DNA from the gel to the nylon membrane.

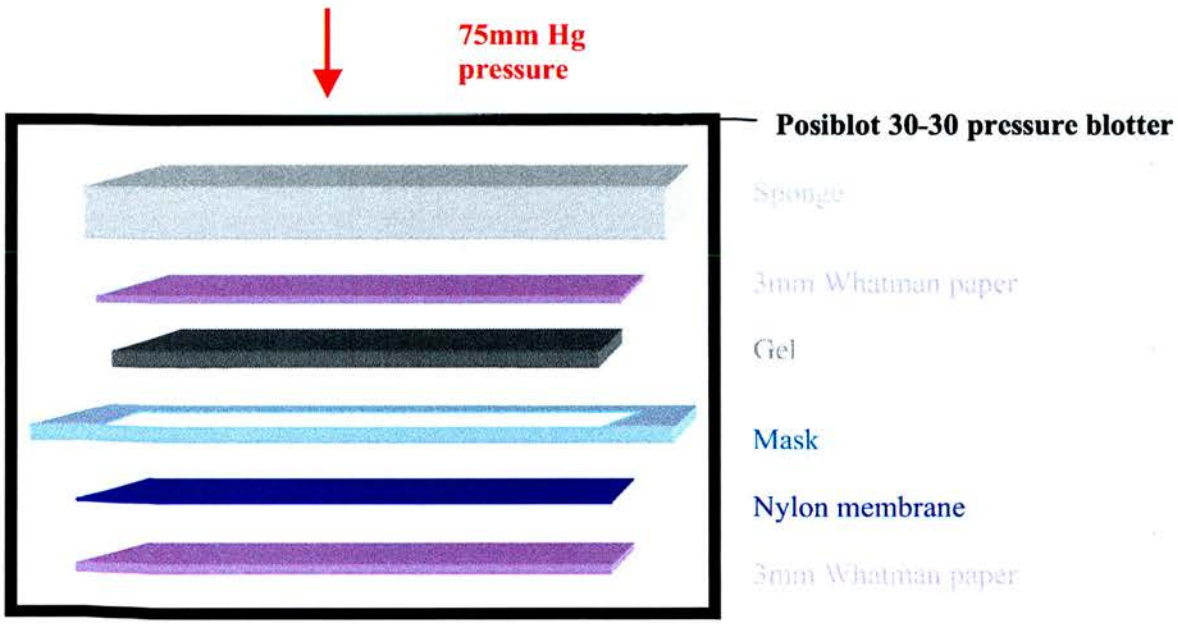

Figure 2.12.1 – Schematic diagram of DNA transfer to a nylon membrane using the Posiblot 30-30 pressure blotter.

After the DNA transfer was complete, the membrane was removed from the apparatus and washed briefly in 2X SSC before being left on Whatman for 15 minutes to allow the membrane to air dry. The DNA was cross-linked to the membrane by exposure to UV light for three minutes and 45 seconds.

2.12.3 Hybridisation and detection

After DNA cross linking the membrane was rolled up length ways and placed in a clean glass tube which had been pre-incubated 55°C in the blotter. Twenty-five ml of pre-hybridisation solution (Table 2.12.2) was added to the cylinder and the membrane incubated with this solution at 55°C for 1 hour.

Reagent	Volume
20X SSC	12.5 ml
10X Blocking reagent (from DIG Wash and Block buffer set)	5 ml
10% SDS	100 µl
30% sarcosine	167 µl
dH_2O	32.23 ml

Table 2.12.2 – Pre-hybridisation solution.

The pre-hybridisation solution was then replaced with 10 ml of the same solution supplemented with 100 pmol of digoxigenin labelled oligonucleotide specific to either SENV D or SENV H (Table 2.12.3) and incubated at 55°C for 3 hours. The membrane was washed twice with 25 ml of 2X SSC with 0.1% SDS and twice with 0.5X SSC with 0.1%SDS, all for 30 minutes at 55°C.

Detection was carried out using the DIG wash and block buffer set (Roche) following the manufacturers instructions. The membrane was washed for 5 minutes in 1X washing buffer before incubation for 30 minutes in blocking solution. A further 30 minutes incubation in 50 ml of blocking solution containing 1.875 units of anti-digoxigenin antibody (Roche) followed by two 15 minutes washes in washing buffer to remove the excess antibody preceded a three minute treatment with detection buffer.

Probe name	Label	Sequence 5'→3'	Reference
SENV D	5' digoxigenin	ATGATAGGCTTCCCYTTTAACTATAACCCA	[Umemura et al., 2001a]
SENV H	5' digoxigenin	CCCCTTCCAGGTATTGCATGAAGAGTATTAC	[Umemura et al., 2001a]

Table 2.12.3 – Hybridisation probes used for HCV secondary real-time PCR.

The membrane was removed from the detection buffer, drained to remove the excess solution and covered evenly with the chemiluminescent substrate CDP-star (Roche). After five minutes, the excess solution was removed and the membrane wrapped in cling-film and placed in a development cartridge with Bio-Max Light film (Kodak) for between 5-25 minutes.

2.13 IgG capture assay

Protein G magnetic beads (Dynal Biotech) were used to capture IgG from a variety of serum samples. The protein G which coats the beads is derived from Group G Streptococci bacteria and has a high affinity for the immunoglobulin IgG. In each case, 50 µl of protein G magnetic beads were washed twice with 0.1 M sodium citrate (pH 5) before the addition of 50 µl of serum. This was incubated at room temperature for 40 minutes with gentle mixing to allow Protein G and IgG to form complexes. The samples were placed on a magnet and the supernatant removed and reserved. The beads were then washed three times with 0.1 M sodium citrate (pH 5) to purify the IgG. Elution of the antibody was performed using 0.1 M sodium citrate buffer with a lower pH (pH 2). This elution process was carried out twice with 30 µl of buffer. Finally, the magnet was used again to capture the beads allowing the removal of the supernatant containing the IgG from the serum sample. The supernatant and eluate from the IgG capture process was extracted using QIAamp Minelute Virus Spin kit (section 2.4.4) and the titre of virus quantified using limiting dilution (section 2.5.2).

2.14 Statistical analysis.

Statistical analysis of samples was carried out using SPSS for Macintosh. A variety of tests were used to analyse these data however, in all cases a P value of less than 0.05 was considered to be significant.

2.14.1 Spearman's rank correlation coefficient.

Spearman's Rank Order Correlation Coefficient was used as a non-parametric measure of the strength of relationship between two variables. It gives the correlation coefficient r_s, an indication if the two variables have a positive correlation, a negative correlation or no correlation, and the P-value associated with the r_s. The P-value predicts how likely it is that the given correlation coefficient would be generated by a random sample. In order to compare the variables, each value is given a rank to compare it with the other variables within the group.

2.14.2 Mann-Whitney U test

The Mann-Whitney U test, also known as the Wilcoxon-Mann-Whitney test is a non-parametric test which assess whether the differences in medians between two observed distributions is normal. The null hypothesis that there is no difference between the medians of two sets of data from unpaired samples.

2.14.3 Wilcoxon signed ranks test

The Wilcoxon Signed Ranks test is the non-parametric equivalent of the paired T-test, which tests a hypothesis about the median of a population distribution. It can be used to test the significance of treatment or experimental manipulation on a population.

2.14.4 Kruskal-Wallis test

The Kruskal-Wallis test is a non-parametric test to compare three or more samples. It is used to test the null hypothesis that all populations have identical distributions and thus the means are equal.

2.14.5 Linear regression

Linear regression is a statistical tool which attempts to model a relationship between two variables. In this test, a linear equation is fitted to a set of data points in order to measure the effect of a single variable.

Chapter 3

Characterisation of Anelloviruses in the Blood Donor Population

3 CHARACTERISATION OF ANELLOVIRUSES IN THE BLOOD DONOR POPULATION

3.1 Introduction

After the discovery of TTV in 1997 efforts were made to assess the prevalence of this virus within the blood donor population. A preliminary study of the Scottish blood donors showed just 1.9% of individuals had detectable TTV DNA in their serum [Simmonds et al., 1998], however this figure was based on a PCR assay using primers from the heterogeneous N22 region of the genome. It is widely accepted that as primers from this region only amplify a limited range to TTV genotypes.

There is no standardised PCR protocol for the detection of TTV but the use of primers from the relatively conserved UTR region of the genome allows for amplification of the all the known TTV genotypes. The UTR of TLMV is sufficiently conserved with TTV to allow the simultaneous amplification of both viruses with some of the UTR based primers, however the viruses are rarely differentiated in literature.

Two SENV genotypes, SENV D and H, are frequently associated with post transfusion non A-E hepatitis although the clinical significance of these viruses in the disease process has yet to be ascertained [Umemura et al., 2001b]. There is limited data concerning the prevalence of these two variants in Europe with most epidemiological data from Eastern Asia where there appears to be a higher prevalence of SENV D than SENV H in almost all the populations studied [Kao et al., 2002; Sugiura et al., 2004; Umemura et al., 2003]. In contrast, data from blood donors in Germany and the USA demonstrate a higher prevalence of SENV H than SENV D [Umemura et al., 2003].

This chapter describes the characterisation of Anelloviruses in Scottish blood donors. The prevalence of TTV and TLMV is updated using primers from the UTR and the frequency of SENV D and H infection is ascertained for the first time.

3.2 Results

3.2.1 Differentiation of Anelloviruses on the Roche Lightcycler

3.2.1.1 UTR PCR

Sequence analysis of both TTV and TLMV has shown regions of the genome which remain partially conserved amongst all the known genotypes. This untranslated region was chosen as the site for PCR primers to ensure the maximum number of viral genotypes were amplified.

Ten HCV/HBV/HIV PCR negative minipools, each consisting of 95 individual Scottish donor plasma samples, were tested using a hemi nested PCR consisting of a first round using primers CVOS/CVOA and second round with primers CVOS/CVIA. All ten of the samples contained a visible band of around 100bp when run on an agarose gel (Figure 3.2.1).

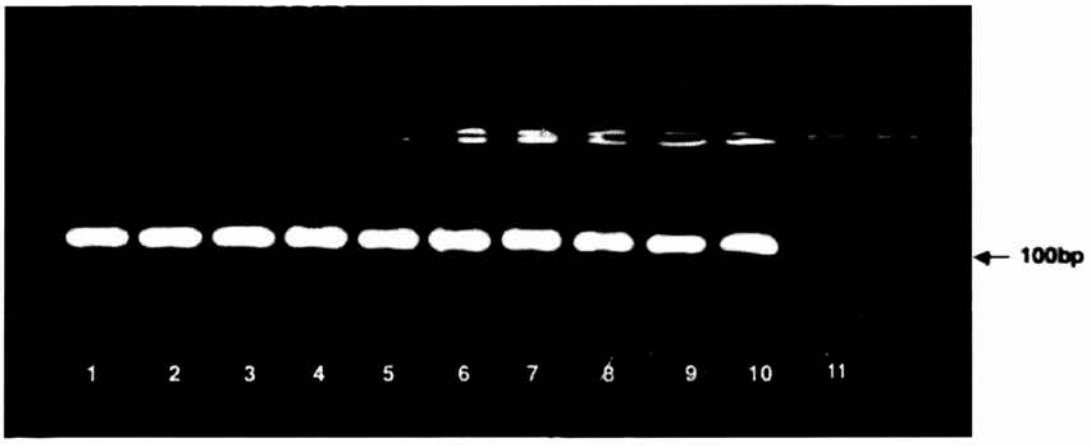

Figure 3.2.1 – PCR for Anelloviruses carried out using serum from 10 minipool plasma samples. Lanes 1-10: minipool plasma secondary PCR product; lane 11: negative control (water).

3.2.1.2 Characterising products of UTR primers on a Roche Lightcycler

Two μl of the primary product from each of the 10 minipool samples used in the standard UTR PCR amplification reaction were added to a secondary reaction carried out on the Roche Lightcycler using the fluorescent dye SYBR green. Amplification was carried out for 40 cycles and a product was obtained for all 10 of the samples as shown in Figure 3.2.2. The products were then characterised by melting curve analysis.

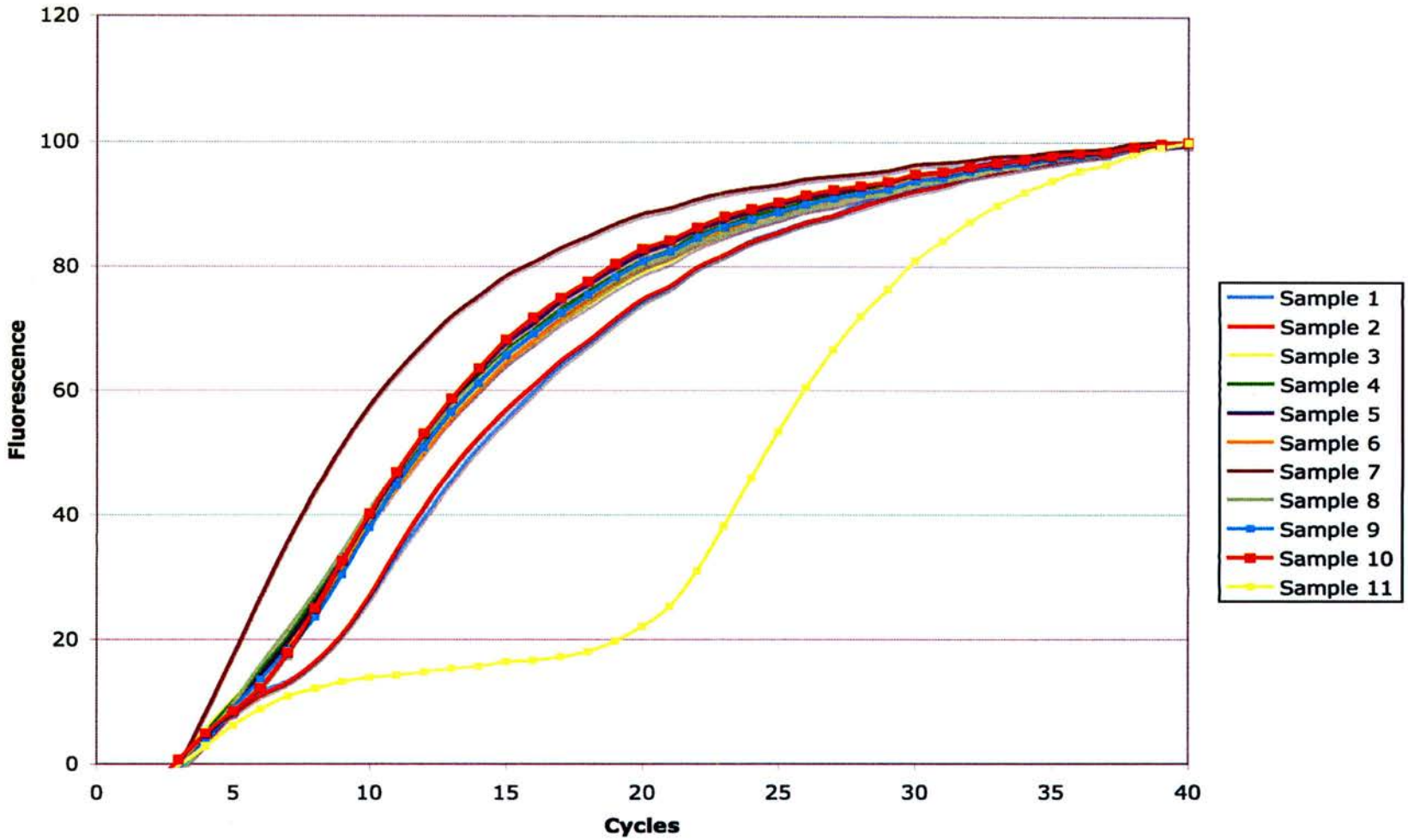

Figure 3.2.2 – Amplification profile of 10 minipool plasma samples amplified with UTR primers and analysed on the Roche Lightcycler. Samples 1-10: minipool plasma; Sample 11: negative control (water).

The melting temperature (Tm) for each sample was calculated by the rate of change in fluorescence with temperature (-*d*(F1/*d*T). The amplicons fell into two different distributions of Tms when characterised, as shown in Figure 3.2.5. It was hypothesised that these corresponded to the two Anelloviruses (TTV and TLMV).

3.2.1.3 Differentiating Tms of PCR products

Based on the supposition that the two distributions of Tms correspond to the two known Anelloviruses, primers were designed to amplify sequences specific to TTV or TLMV. Twenty HIV/HBV/HCV PCR negative plasma samples were amplified with primer CVOA and either TTV-OS for the TTV specific PCR or TLMV-OS to detect TLMV. The secondary PCR was carried out with primers CVOS and CVIA.

Of the 20 samples, six were PCR positive using the TTV specific primer (Figure 3.2.3), two were positive using the TLMV specific primer (Figure 3.2.4) and four were positive using both primers as shown in Table 3.2.1.

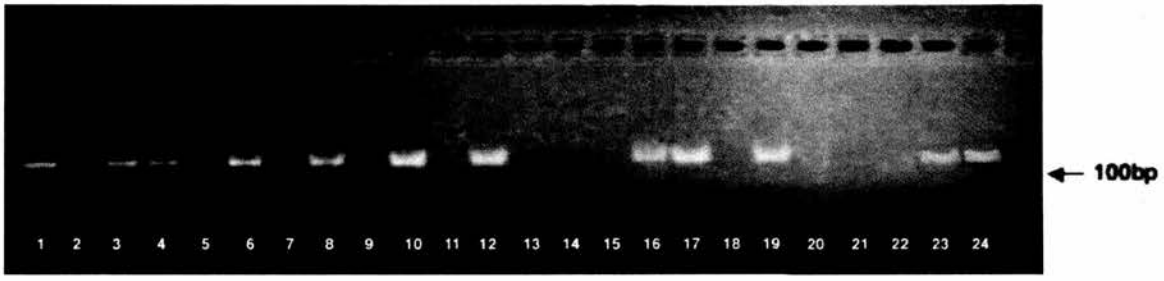

Figure 3.2.3 – PCR using TTV specific primers on plasma from 20 Scottish blood donors. Lanes 1-20: Secondary PCR product from plasma samples; Lanes 20 & 21; Negative control (water); Lanes 23 & 24: Positive control (minipools).

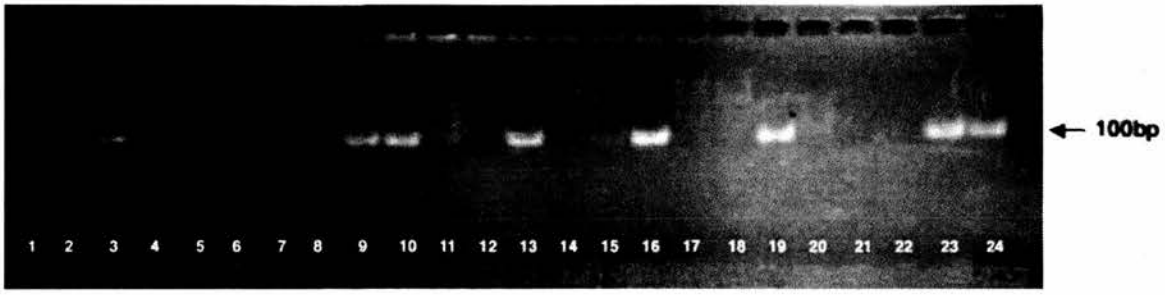

Figure 3.2.4 - PCR using TLMV specific primers on plasma from 20 Scottish blood donors. Lanes 1-20: Secondary PCR product from plasma samples; Lanes 20 & 21; Negative control (water); Lanes 23 & 24: Positive control (minipools).

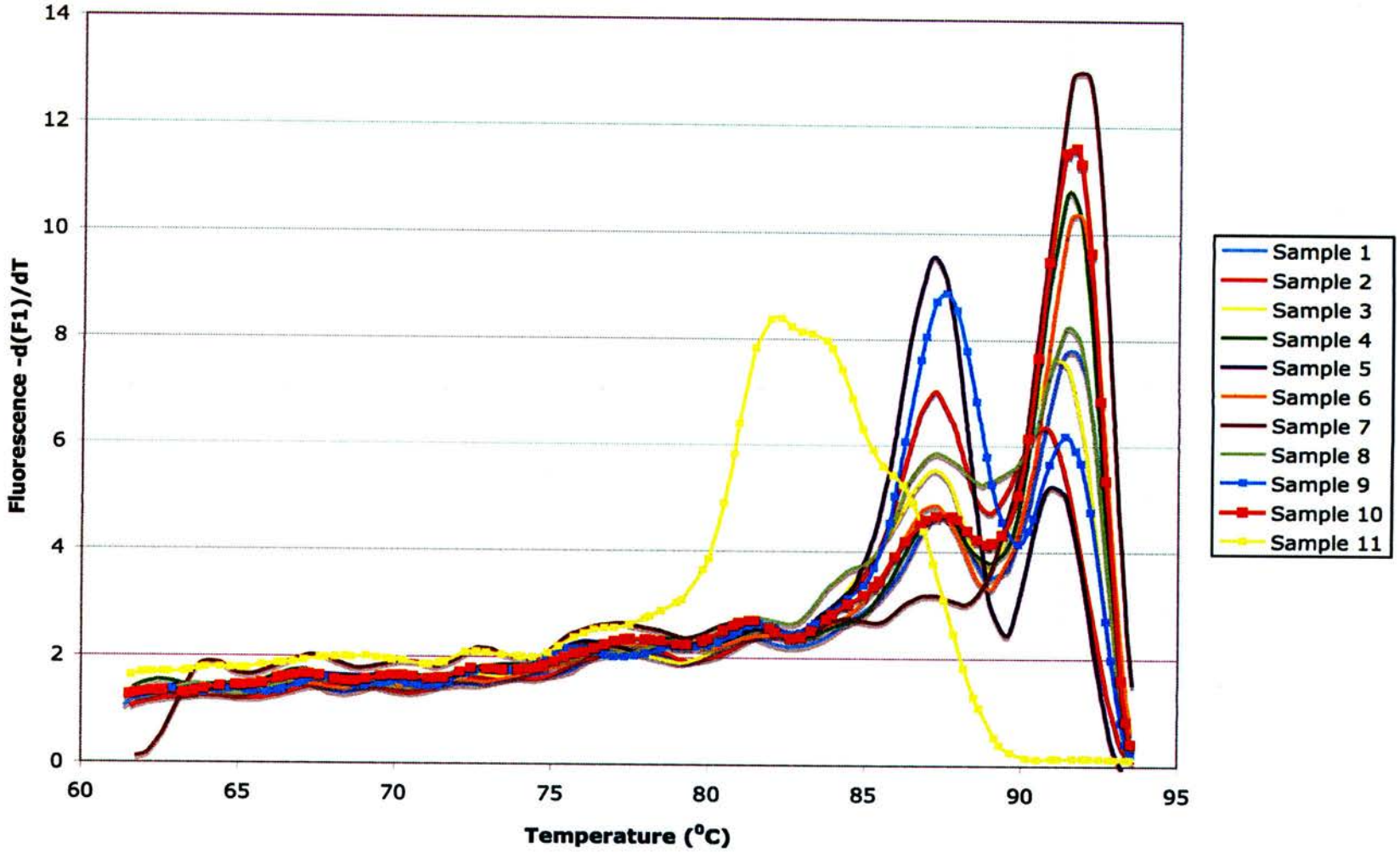

Figure 3.2.5 - –Melting cure profiles of 10 minipool plasma samples amplified with UTR primers and analysed on the Roche Lightcycler. Samples 1-10: minipool plasma; Sample 11: negative control (water).

Sample	TTV specific primers	TLMV specific primers
1	+	-
2	-	-
3	+	+
4	+	-
5	-	-
6	+	-
7	-	-
8	+	-
9	- (+)	+
10	+	+
11	-	-
12	+	-
13	-	+
14	- (+)	-
15	- (+)	- (+)
16	+	+
17	+	-
18	-	-
19	+	+
20	-	-

Table 3.2.1 – Detection of viruses in plasma samples using TTV and TLMV specific primers analysed on an ethidium bromide gel. Samples gave a different result when analysed on a Roche Lightcycler are shown in brackets.

The primary product from each sample amplified with TTV and TLMV specific primers was subjected to a secondary nested PCR on a Lightcycler using the generic internal primers CVOS/CVIA.

Of the 20 samples analysed on the Lightcycler, seven were PCR positive using the TTV specific primer, one with the TLMV specific primer and six were positive with both primers. The increased incidence of PCR positive samples when using the Lightcycler could be because this is a more sensitive detection format than agarose gel electrophoresis and the one-step secondary PCR and analysis method utilised by the Lightcycler cuts down on operator error.

When the plasma amplified with the TTV specific products was analysed using a melting curve on the Lightcycler (Figure 3.2.6) they showed Tms with a median value of 91.06°C (range 89.4-92.02°C). This appears to correspond to the higher distribution of values seen in 3.2.1.2. The median Tm for the samples that were amplified using a TLMV specific primer (Figure 3.2.7) was 86.79°C (range 85.47-87.56°C), which corresponds with the lower distribution of Tms seen with the UTR primers.

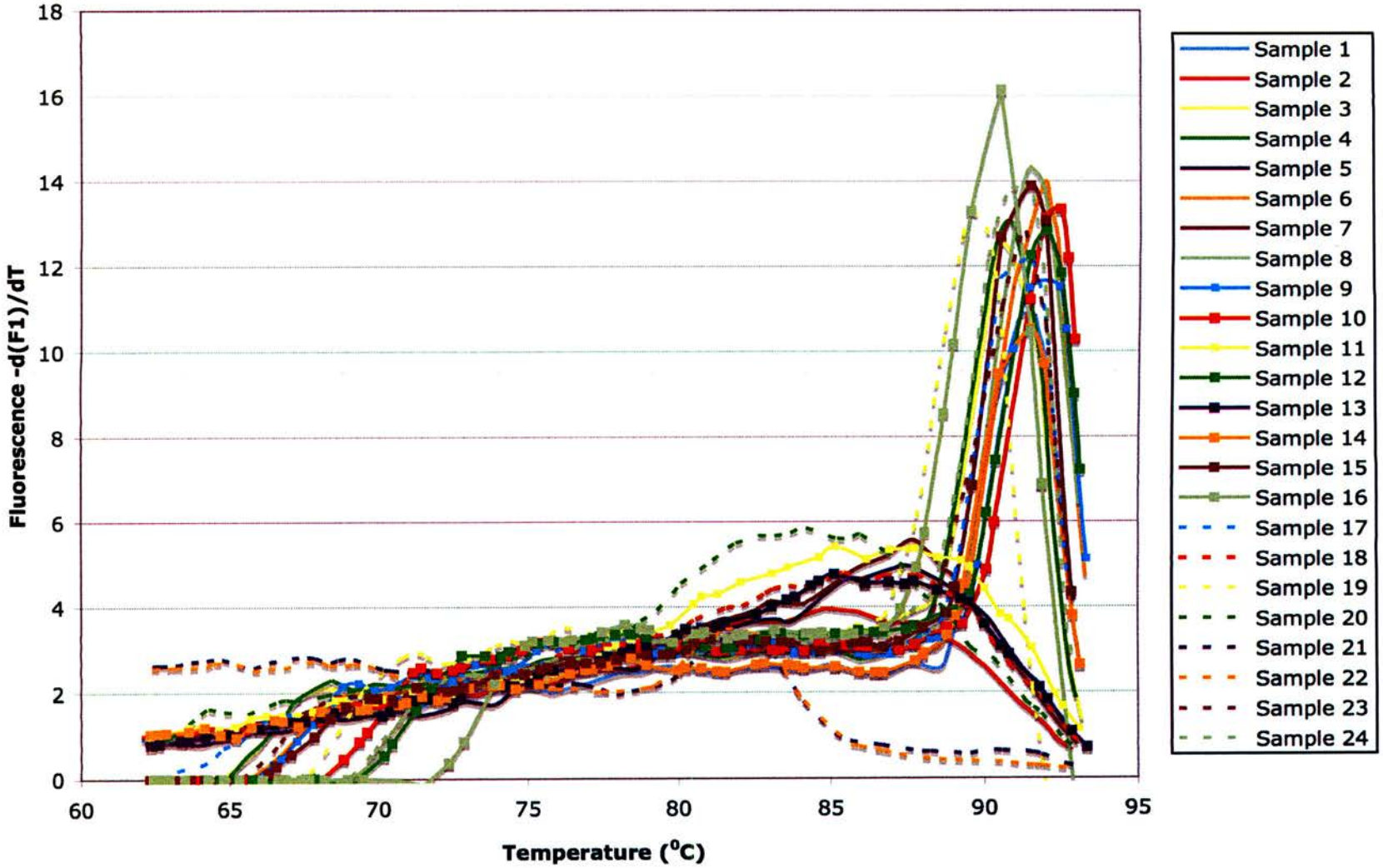

Figure 3.2.6 – Melting curve profiles of products amplified using TTV specific primers and analysed on a Roche Lightcycler. Samples 1-20: plasma samples from Scottish blood donors; Samples 21 & 22: negative controls (water); Samples 23 & 24: positive controls (minipools).

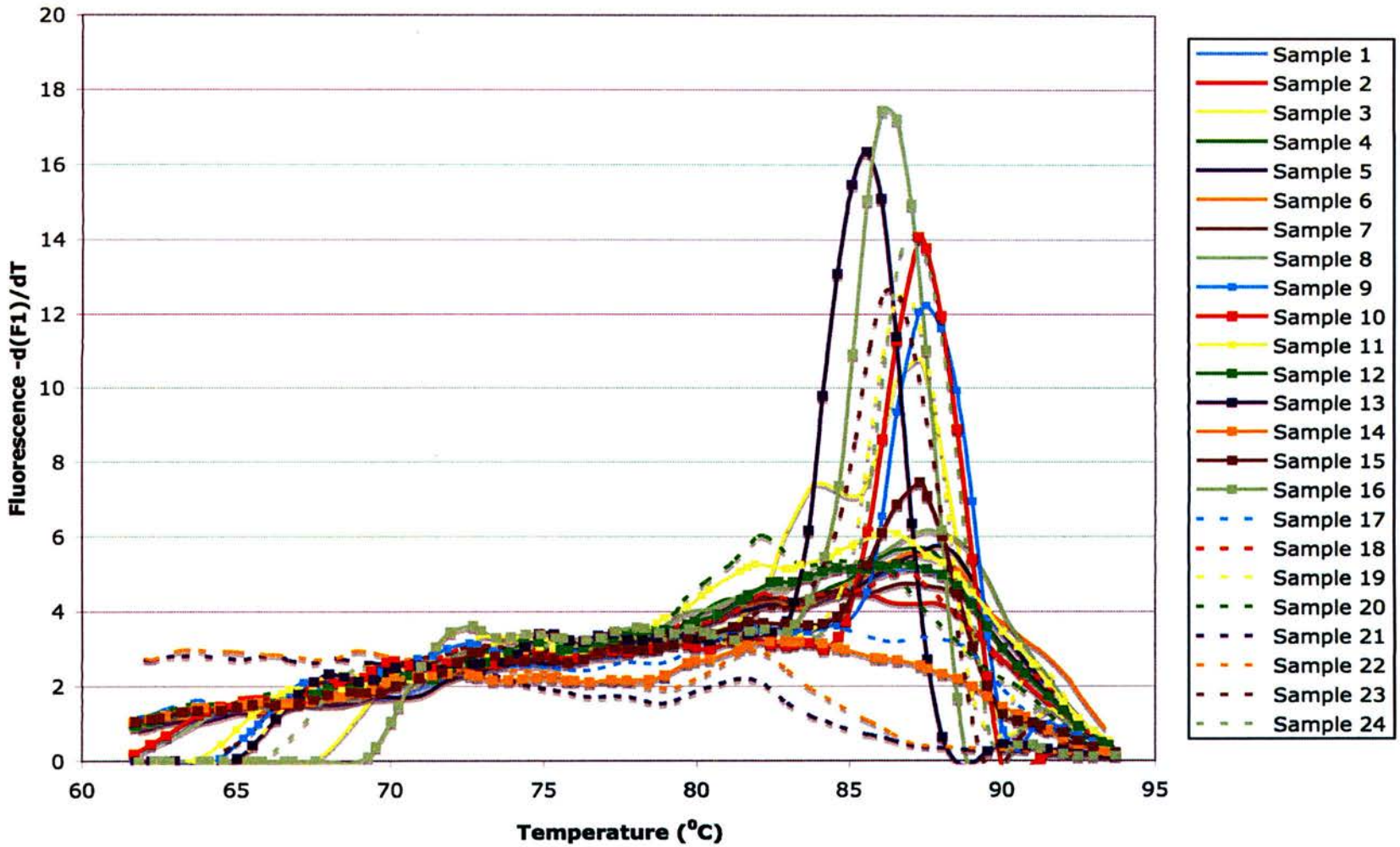

Figure 3.2.7 – Melting curve profiles of products amplified using TLMV specific primers and analysed on a Roche Lightcycler. Samples 1-20: plasma samples from Scottish blood donors; Samples 21 & 22: negative controls (water); Samples 23 & 24: positive controls (minipools).

3.2.1.4 Cloning and sequencing products from TTV and TLMV specific PCR

To identify the products amplified with the virus specific primers conclusively, four plasma samples positive with the TTV specific primer and four samples positive with the TLMV specific primer were cloned into a vector and sequenced.

Eleven sequences from TTV specific primers and 15 from TLMV specific primers were compared with prototype TTV and TLMV sequences and other previously published sequences submitted to GenBank (Figure 3.2.8). In both cases the sequences obtained from the clones closely matched the known TTV and TLMV sequences.

The clones used in the sequencing reaction were re-amplified on the Lightcycler for a final time and melting curve analysis performed to determine their Tm (Figure 3.2.9). The TTV-like sequences correspond to the distribution of amplicons with the higher Tms and the TLMV-like sequences with the lower melting temperature distribution. Analysis of the sequences suggested the differences in Tms between the two viruses might be down to two factors. Firstly, the TTV sequences are generally 2-4 bases longer in the UTR region in which the PCR primers are sited. The TTV sequences also have a higher guanine + cytosine (G+C) base content in the region of interest, with a mean G+C value of 65.4% compared with 58.6% G+C composition in the comparable region of TLMV. Both these facets will increase the binding strength of the amplicon, contributing to the higher temperature needed to melt the TTV product.

Two of the clones, TLMV 1/1 and 1/2 showed a lower Tm (83.7°C) than the other products amplified with the TLMV specific primer (median value 87.14°C, range 85.9-88.4°C). When the corresponding sequence was analysed, it contained a five nucleotide deletion not present in the other TLMV sequences. This is further evidence of the influence of amplicon length on melting temperature.

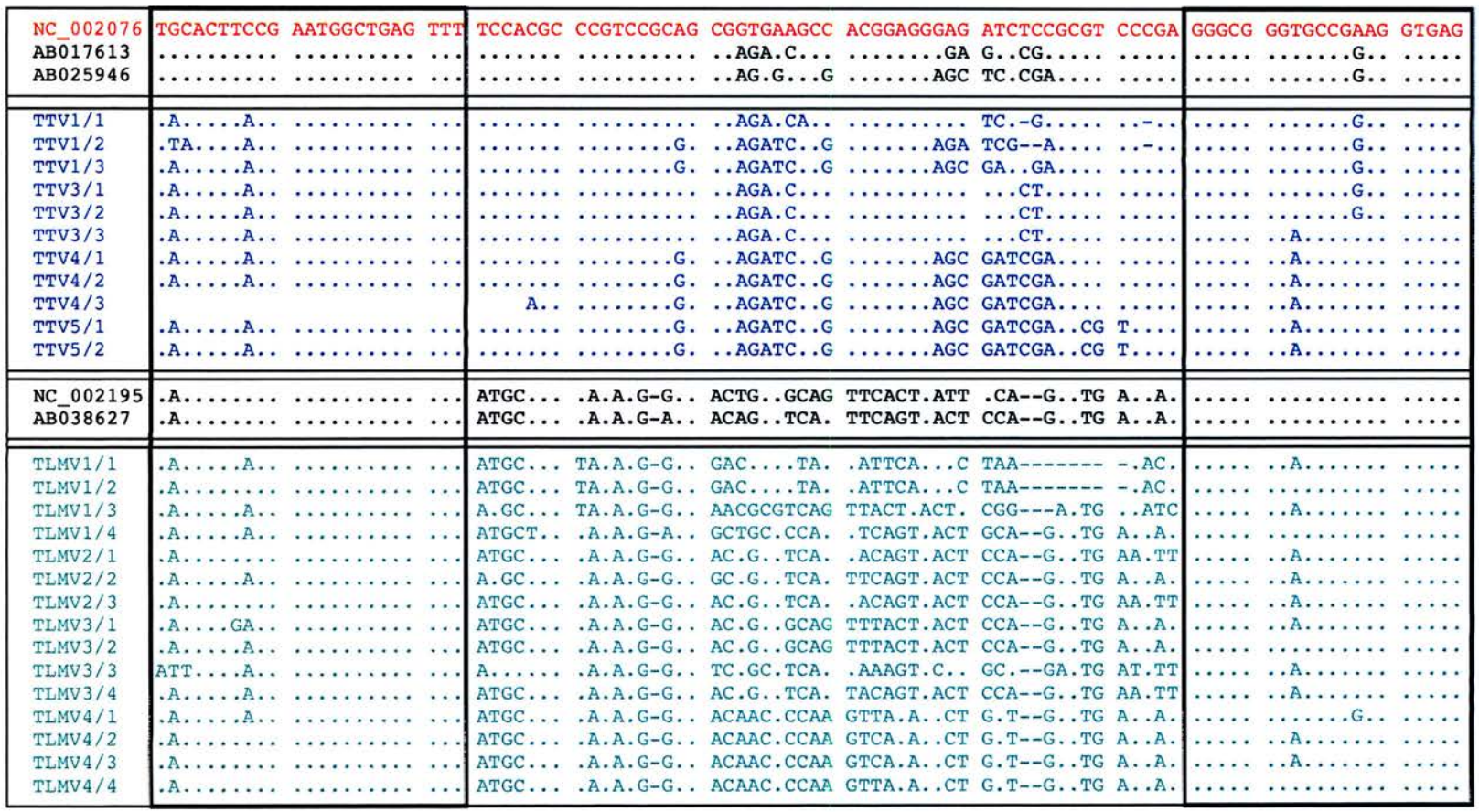

Figure 3.2.8 – Sequences obtained from cloning and sequencing products amplified using virus specific primers TTV-OS (TTV 1 & TTV 3-5, clone number added as suffix, shown in blue) and TLMV-OS (TLMV 1-4, shown in green) relative to the TTV sequence NC_002076 (shown in red). Previously published sequences are indicated by accession numbers and shown in black. Symbols: . , sequence identical to prototype TTV sequence; - ,gap introduced to preserve the alignment of homologous nucleotides.

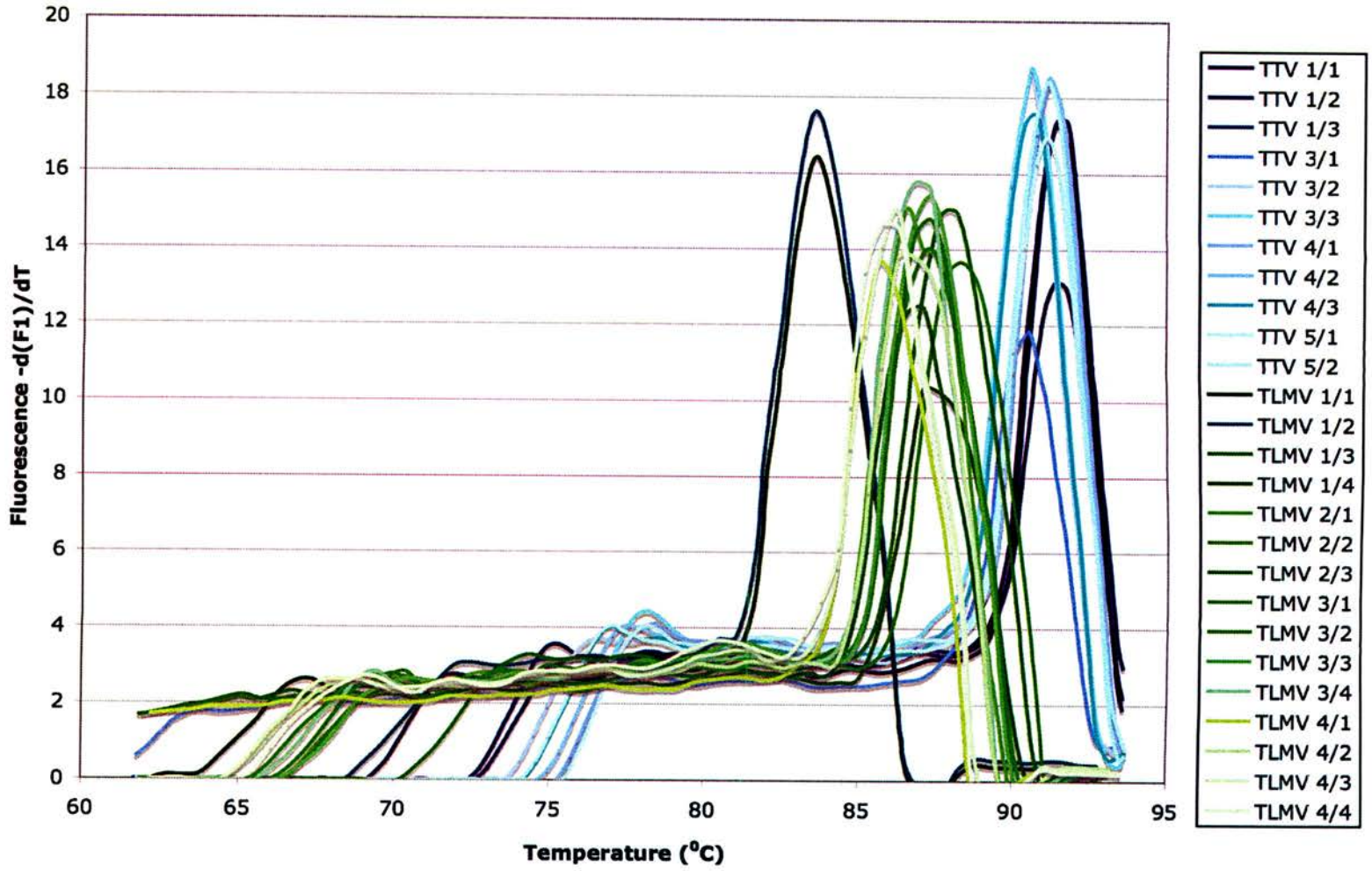

Figure 3.2.9 – Melting peak profiles of molecular clones used to sequence products from TTV (shown in blue) and TLMV (shown in green) specific PCRs.

3.2.2 Prevalence of Anelloviruses in the Scottish blood donor population

3.2.2.1 Prevalence of TTV and TLMV in Scottish blood donors

Using the method of differentiating TTV and TLMV described in 3.2.1, 100 plasma samples from HIV/HBV/HCV negative Scottish blood donors, a low risk adult population, were screened. After PCR with the outer primer set CVOA/CVOS, a hemi nested PCR was carried out on the Roche Lightcycler. The melting temperature for each sample was analysed and the presence of TTV and/or TLMV in the sample determined.

Of the 100 samples tested, 88 were PCR positive. Using the Tms of the products, these 88 could be categorised into those that were TTV positive (34/88, 38.6%), those that were TLMV positive (18/88, 20.5%) and those that contained a heterogeneous mix of both viruses (36/88, 40.9%). The distribution of Tms within these PCR positive samples is shown in Figure 3.2.10.

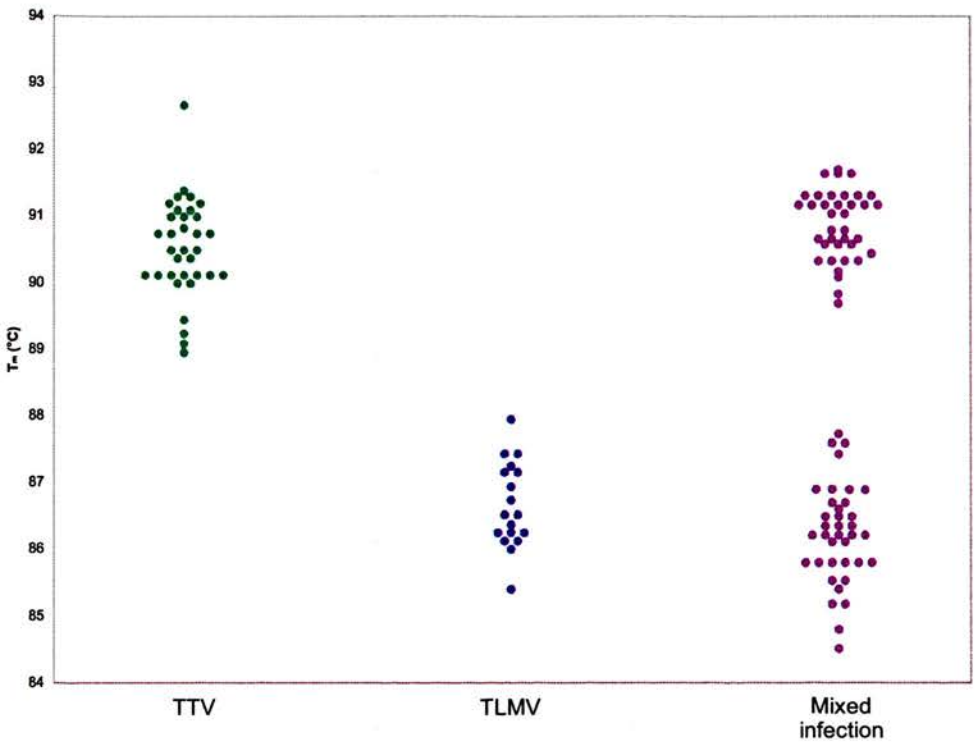

Figure 3.2.10 – Distribution of Tm values from 88 TTV/TLMV PCR positive Scottish blood donors produced by melting curve analysis on the Roche Lightcycler.

3.2.2.1.1 Comparison of UTR and N22 primers

The prevalence of Anellovirus infection varies greatly between published studies. This is partially explained by the lack of consistency when investigators chose PCR primers. To compare the frequency of virus infection identified with different primer sets, 100 blood donor samples were amplified with the UTR primers described earlier as well as with a set of hemi- nested primers situated in the N22 region [Okamoto, 1998] and visualised on an ethidium bromide stained agarose gel. Of the 100 samples tested, 88 were positive using the UTR primers and five with the N22 primers. This confirms that studies using the N22 primers as the basis for their survey are likely to be underestimating the prevalence of these viruses.

3.2.2.2 Prevalence of SENV D and SENV H in Scottish blood donors

A one round PCR reaction was carried out on 15 Scottish blood donor minipools samples with SENV D and SENV H specific sense primers (D10S and C5S) and a generic anti-sense primer (L2AS) [Umemura et al., 2001a]. The resulting product was visualised by gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide, however no bands of the correct size were visible.

The process was repeated and, after gel electrophoresis, the DNA transferred onto a membrane and probed with SENV D and SENV H specific digoxigenin labelled probes. This yielded positive signals for 0/15 samples for SENV D and 5/15 for SENV H (roughly equivalent to five positive donors in 1425 samples). This was a significantly lower level of infection than expected so, in order to increase the sensitivity of the PCR, a set of outer primers were designed to amplify a portion of SENV D/H that lies outwith the amplicon produced by the published primers. Fifteen minipools samples were subjected to a nested PCR using the new outer primers followed by a SENV D and H specific inner PCR and Southern blotting with genotype specific digoxigenin labelled probe. The results of this were compared with Southern blots carried out on the DNA from a one round PCR reaction.

As Figure 3.2.11 shows, no virus was detected in any of the samples after one round of PCR however the improved sensitivity of the nested reaction revealed 3/15 were SENV D PCR positive (Figure 3.2.12). Although the one round SENV H Southern blot (Figure 3.2.13) detected virus in 5/15 minipools, the inclusion of the outer primers increased the sensitivity and in the nested PCR 15/15 samples were positive (Figure 3.2.14).

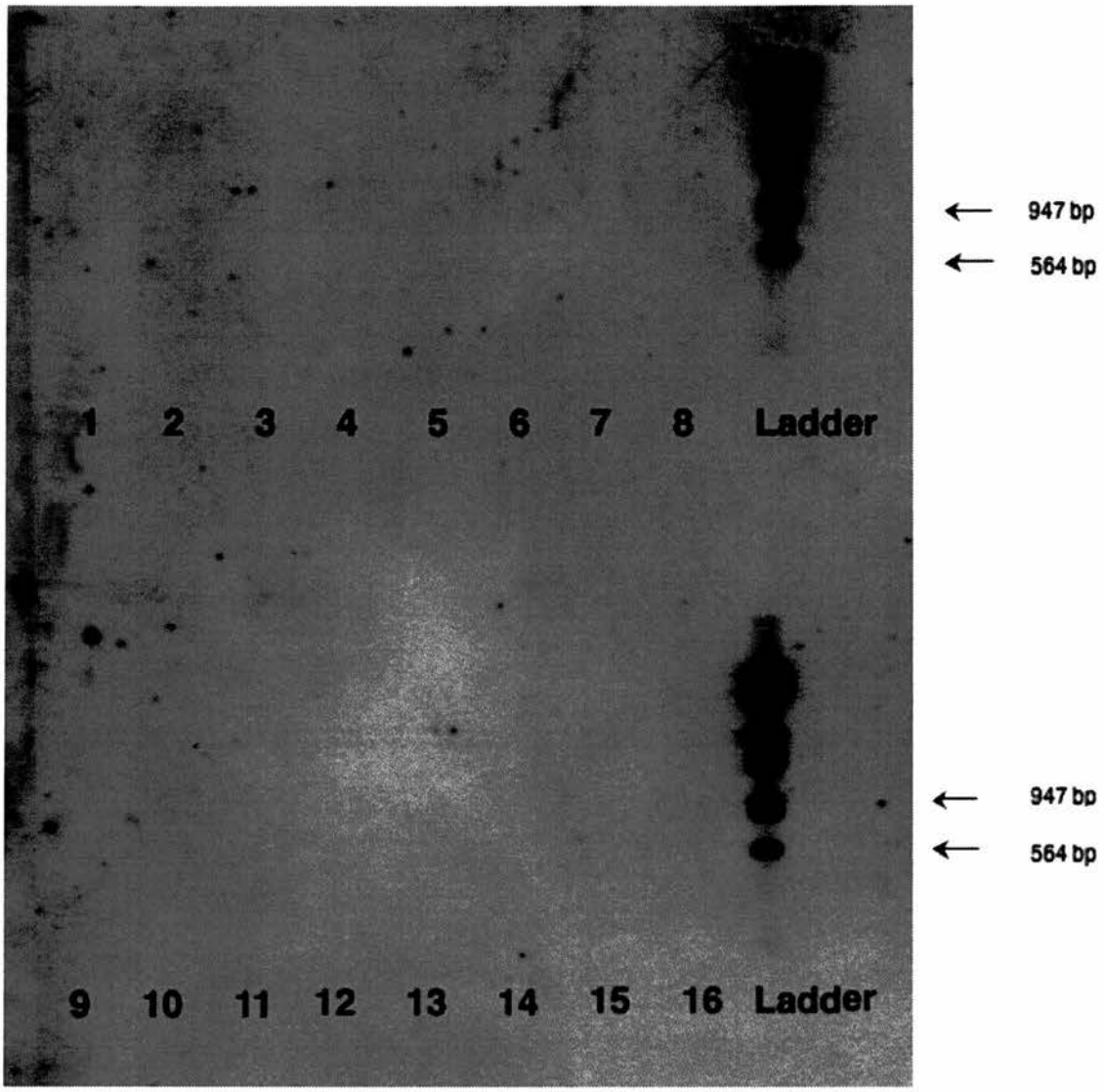

Figure 3.2.11 – Southern blot of one round SENV D PCR (primers: D10S and L2AS). Lanes 1-15: Scottish blood donor minipools 1-15; Lane 16: Negative control (water); Ladder: Digoxigenin labelled molecular weight marker.

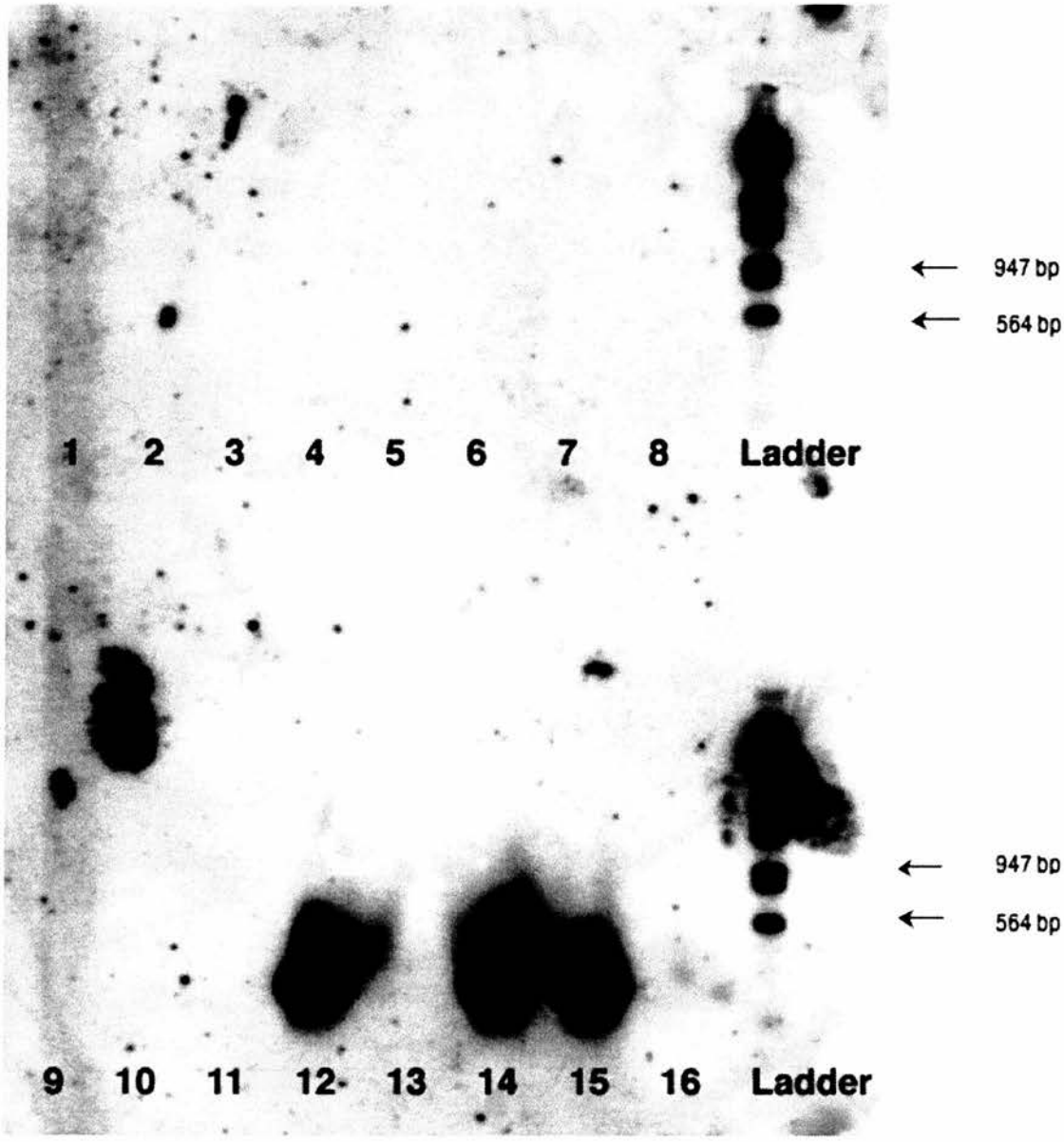

Figure 3.2.12 – Southern blot of nested SENV D PCR (primers: first round: SENV anti-sense and SENV sense; second round: D10S and L2AS). Lanes 1-15: Scottish blood donor minipools 1-15; Lane 16: Negative control (water); Ladder: Digoxigenin labelled molecular weight marker.

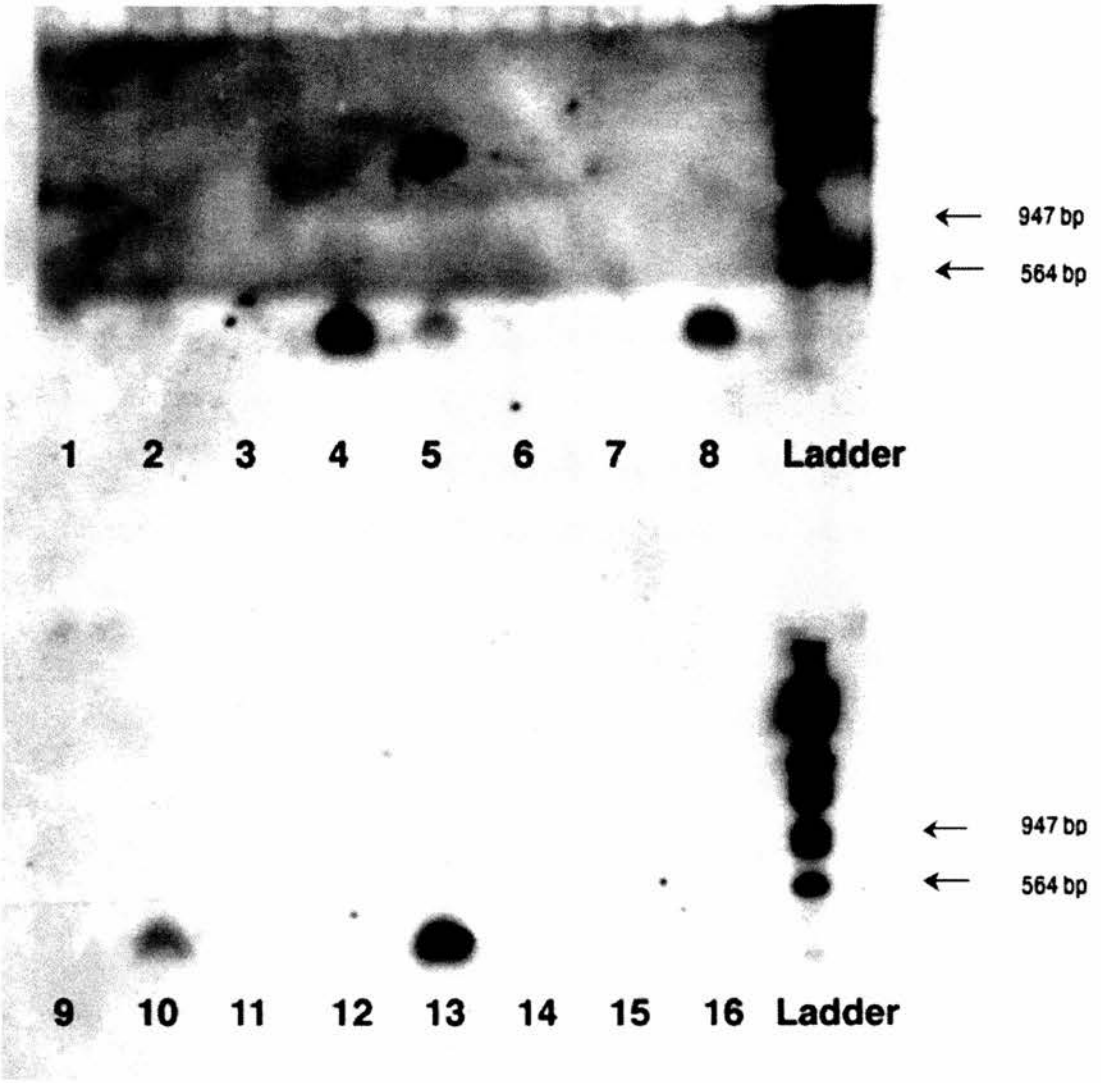

Figure 3.2.13 – Southern blot of one round SENV H PCR (primers: C5S and L2AS). Lanes 1-15: Scottish blood donor minipools 1-15; Lane 16: Negative control (water); Ladder: Digoxigenin labelled molecular weight marker.

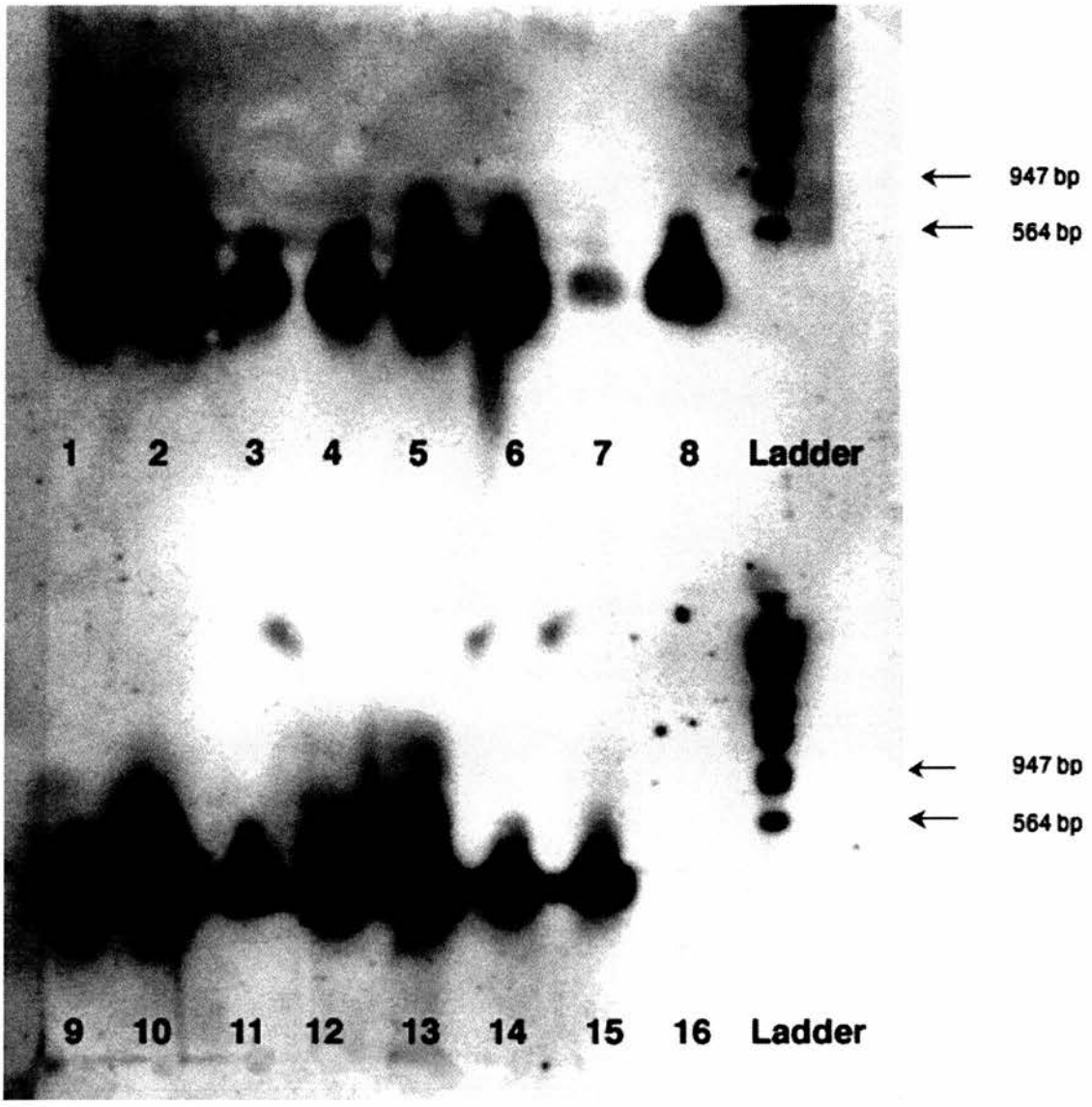

Figure 3.2.14 – Southern blot of nested SENV H PCR (primers: first round: SENV anti-sense and SENV sense; second round: C5S and L2AS). Lanes 1-15: Scottish blood donor minipools 1-15; Lane 16: Negative control (water); Ladder: Digoxigenin labelled molecular weight marker.

The cross reactivity of the probes used for Southern blotting was investigated by probing SENV D PCR product with SENV H probe and visa versa. No cross reactivity was found (data not shown).

A total of 192 individual plasma samples from Scottish blood donors were tested for the presence of SENV D and SENV H DNA by the method described. Of those samples, 1/192 contained SENV D DNA (0.5%), 21/192 samples contained SENV H DNA (10.9%) and 2/192 contained both SENV D and SENV H (1%).

The prevalence of SENV D in the Scottish blood donor population was significantly lower than data collected from other countries. To confirm the discrepancy between the level of SENV D in Scotland and the higher prevalence seen in Asia was a result of geographical differences in virus distribution, fifteen minipools from Taiwanese blood donors (each containing plasma from 95 individual donations) were tested for the presence of SENV D. All fifteen of the samples were positive for SENV D DNA (data not shown).

3.3 Discussion

The extreme genetic diversity of TTV and TLMV has hampered the development of accurate methods of determining the prevalence of these viruses. Fortunately, regions of conservation within the UTR were detected which have been instrumental in the discovery of the ubiquitous nature of TTV, even in healthy populations. The primer sites lie in close proximity to sequences known to associate with transcriptional machinery and the high degree of conservation within this region is probably the result of functional constraints [Hijikata et al., 1999].

An alignment of published human TTV and TLMV sequences was used to design primers which were capable of amplifying TTV and TLMV simultaneously from a heterogeneous population of viruses within a single sample. This method was used to screen a cohort of healthy Scottish blood donors for the presence of TTV and TLMV. It was found that 88% of blood donors were infected with an Anellovirus, confirming the high prevalence of infection reported in other countries [Biagini and de Micco, 2006; Huang et al., 2001].

Analysis of the Tms of the PCR products using melting curve analysis allows for fast and accurate differentiation of TTV and TLMV from a single sample. Sequence analysis determined this was possible because of both length polymorphisms between TTV and TLMV and differences in the proportions of guanine and cytosine base pairs. The TTV sequences were determined to be between two and four bases longer than the TLMV sequences with a mean G+C content of 65.4% for TTV and 58.6% for TLMV. G+C base pairing results three hydrogen bonds compared with two bonds when adenine and thymine (A+T) are paired. This increases the stability of G+C pairs, which in turn increases the temperature needed to disrupt the bonding and denature the DNA.

By differentiation of the Anelloviruses in Scottish blood donors, it was revealed that 34% were infected with TTV, 18% with TLMV and 36% with a heterogeneous mixture of both viruses. This indicates that 70% of the Scottish blood donor population are infected with

TTV and 54% with TLMV. In 2006 a study investigating the prevalence of Anelloviruses in French blood donors reported findings similar those described here, with a total prevalence of 88%, 66% infected with TTV, 62% with TLMV and 44% with mixed TTV and TLMV infection [Biagini and de Micco, 2006]. Although the presence of TTV and TLMV in any of the samples cannot be ruled out as low titres of virus might be below the level of detection of the assay, these data show that TTV and TLMV are equally prevalent and both viruses can replicate and co-exist in a large proportion of apparently healthy individuals.

The N22 region derived from ORF1 of TTV has been the target of the majority of PCR based assays and, although they have been greatly improved, amplification with primers situated in this region is restricted to TTV genotypes 1-6 [Itoh et al., 1999]. To compare the detection of TTV in plasma with different primer sets, the same 100 blood donor samples that were used to assess the prevalence of TTV using the UTR primers were tested with N22 primers. Only 5% of the samples were PCR positive using this method, suggesting a low prevalence of genotypes 1-6 within the Scottish blood donor population. This also reinforces the discrepancy in sensitivity between the two sets of primers, signifying that studies using the N22 method to assess the incidence of TTV infection are likely to underestimate the true frequency of the population.

SENV D and H are found in a higher percentage of patients with fulminant hepatitis than in healthy blood donors, which has led to the hypothesis that these viruses might play a role in liver diseases [Sugiura et al., 2004; Tangkijvanich et al., 2003]. There is a lack of prevalence data for these viruses in the Western world and, given the putative pathogenic character of these viruses, an investigation into the frequency of infection in Scottish blood donors was appropriate. SENV D was found at low frequencies in the population studied, with only 0.5% of donors infected. A total of 10.9% of the samples had evidence of SENV H DNA and 1% were infected with both SENV D and SENV H. These data contribute to the increasing evidence of the global distribution of these viruses. Interestingly, the high level of SENV H compared to SENV D in Scottish blood donors is the reverse of what is observed in healthy adults and blood donors in China, Greece, Japan and Taiwan [Kao et al., 2002; Mu et al., 2004; Umemura et al., 2003]. It is not known why this is the case, however it could be the route of transmission differs between the subtypes.

As with TTV and TLMV, the presence of SENV D and H in apparently healthy individuals suggests they are not pathogenic *per se*. Many studies use blood donors as a control group

for SENV infection in patients with hepatitis and liver disease, however it has been suggested that because blood donors are generally younger, from a high socioeconomic group and without exposure to injection drug use it is inappropriate to use them in this way. Using patients with non-viral liver diseases such as autoimmune hepatitis and primary biliary cirrhosis as controls, it was found there was no significant increase in the prevalence of SENV in individuals with cryptogenic hepatitis [Shibata et al., 2001]. Kao and colleagues also showed that although SENV D and H infection did appear to be associated with a slight increase in serum ALT levels, it was not found to be significant. They also reported that individuals with dual SENV/HCV or SENV/HBV infection did not have ALT levels significantly higher than those infected solely with HCV and HBV [Kao et al., 2002]. This suggests that SENV D and H infection, even in conjunction with hepatitis inducing virus, does not increase the extent of the liver damage.

In conclusion, using an assay developed in the course of this project, this survey has demonstrated a high prevalence of TTV and TLMV infection in Scottish blood donors. The frequency of infection of SENV D and H has also been established in Scotland for the first time. A high degree of dual TTV and TLMV infection was observed however, the frequency of infection with TTV genotypes 1-6 and SENV D and H was relatively low. These data contribute to the premise of widespread global distribution of Anelloviruses.

Chapter 4

TTV and TLMV Levels in Tissue Samples

4 TTV AND TLMV LEVELS IN TISSUE SAMPLES

4.1 Introduction

TTV was initially found in the serum of a blood transfusion recipient with non A-E hepatitis and the putative association with hepatitis and liver disease lead to investigations into the titre of TTV in the liver. An early publication reported that TTV DNA was present in the liver at levels 10-100 fold higher than in the serum of the same patient [Okamoto, 1998], contributing to the liver disease association debate. Further investigations discovered that TTV was not restricted to the liver, with detection in extrahepatic tissues including kidney, intestines, spleen and stomach using *in situ* hybridisation [Fan et al., 2000].

It was first hypothesised that TTV may be present and replicating in the bone marrow when a significant decrease in the titre of TTV in serum was observed in the myelosuppressed period following bone marrow transplantation [Kanda et al., 1999]. Subsequently double stranded replicative intermediates were confirmed in bone marrow cells [Okamoto et al., 2000d] however this was not accompanied by any pathology.

The TTV titre in the serum of HIV positive individuals has been the subject of a number of papers [Martinez et al., 2000; Moen et al., 2002b; Pistello et al., 2001] however there is a lack of data on the presence of TTV in the tissues of these individuals. There are several haematological complications of HIV and AIDS, such as thrombocytopenia and neutropenia, whose underlying cause remains somewhat of a mystery. Given the evidence of TTV replication in the bone marrow and the increased titre of TTV in the serum of those infected with HIV, it is possible that TTV will play a role in the unexplained haematological abnormalities seen in HIV positive patients.

In this chapter, the findings of an investigation into the titre of TTV and TLMV in tissues taken from HIV positive and negative individuals are presented and discussed. In the instances of the HIV positive cases, correlation of the titre of Anellovirus with the CD4 count was attempted to assess the role of the immune system in the replication of TTV and TLMV.

4.2 Results

4.2.1 TTV/TLMV titres in HIV positive and HIV negative bone marrow

4.2.1.1 TTV/TLMV titres in HIV positive bone marrow

Firstly, to determine if bone marrow samples were infected with TTV/TLMV, six post mortem, bone marrow samples from individuals who were infected with HIV (two female, four male, mean age 36; range 28-48 years) were provided by Jeanne Bell, Professor of Neuropathology, Western General Hospital, Edinburgh, EH4 2XU and stored at -40°C until required. After DNA extraction using the phenol chloroform method, DNA from each of the samples was added to a PCR that used primers situated in the UTR of TTV and TLMV. All six samples (12, 292, 87, 23, 11 and 8) produced a band of the correct size (≈100bp) and were considered positive for either TTV or TLMV.

To determine the titre of Anellovirus present in each of the samples, limiting dilution PCR was carried out. In short, samples were titrated to an endpoint in eight, serial, ten fold dilution steps before analysis using the UTR PCR method (Figure 4.2.1 & Figure 4.2.2). The concentration of DNA was analysed using a spectrophotometer and the amount of DNA contained in each dilution calculated. The final dilution producing a visible signal was observed and, as it was presumed this contained at least one copy of the target of interest, the titre of virus in the bone marrow sample estimated. The endpoint dilution was not achieved in the first eight dilutions for samples 87 and 23, which were further diluted in order that the last PCR positive dilution could be observed (Figure 4.2.2).

The titre of TTV/TLMV present in each of the six bone marrow samples is summarised in Table 4.2.1 (mean 107 copies per cell, median 1.33 copies per cell). The CD4+ T cell counts for each sample were provided by Jeanne Bell and served as an indicator of the level of immunosuppression of each patient.

The table shows that all six of the HIV positive bone marrow samples were infected with either TTV or TLMV. The viral titres ranged from one viral copy per 200 cells to over 350 copies per single cell. Spearman's rank order coefficient was used to investigate if any correlation between viral titre and CD4+ T cell count could be established. This test produced a p-value of 0.11 indicating there was no significant correlation between these two factors.

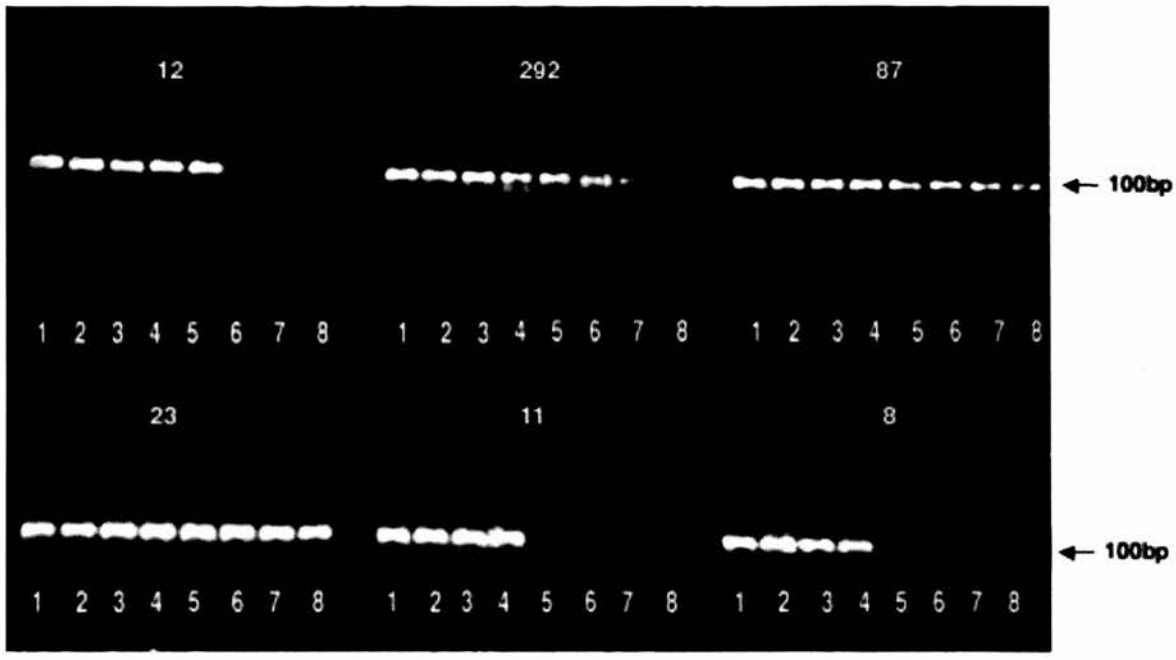

Figure 4.2.1 – Secondary UTR PCR products of DNA extracted from six HIV positive bone marrow samples. Each sample was serially diluted eight times in water. Lane 1: 1 in 10 dilution of bone marrow DNA; Lanes 2-8: Consecutive 1 in 10 dilutions of bone marrow DNA up to 1 in $1x10^8$. Sample numbers are given above each set of dilutions.

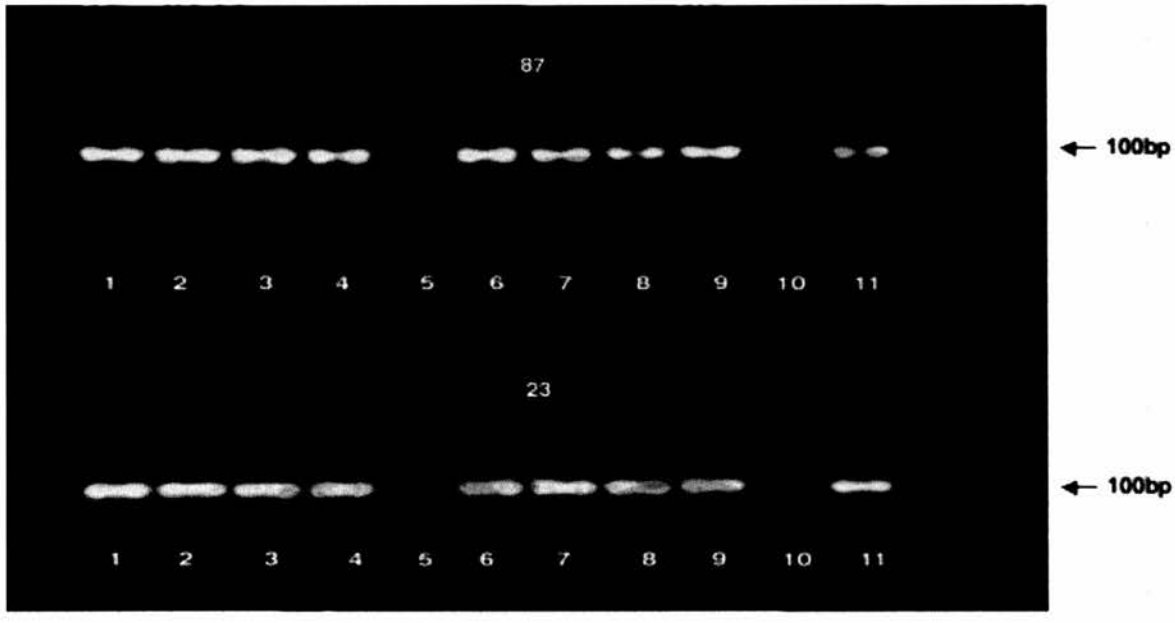

Figure 4.2.2 –Extended limiting dilution PCR for samples 87 and 23. Lanes 1-4, 6-9, 11-12: 10 fold serial dilutions of DNA extracted from HIV positive bone marrow added to the primary PCR reaction (Lane 1; 1 in 10 dilution of DNA in water and further dilutions to Lane 12: 1 in $1x10^{10}$). Lanes 5&10: negative controls (water).

Patient number	Age (years)	Sex (Male/Female)	TTV/TLMV copies per cell (copies)	CD4+ T cell count (cells/mm^3)
12	28	F	0.0371	12
292	43	M	2.63	292
87	48	F	285	87
23	31	M	357	23
11	33	M	0.00471	11
8	34	M	0.0222	8

Table 4.2.1 – Data from post mortem HIV positive bone marrow samples. Viral copies per cell estimated using limiting dilution PCR. CD4+ T cell count provided by Jeanne Bell. The 'copies per cell' data are shown to three significant figures.

4.2.1.1.1 Differentiation of Anelloviruses in HIV positive bone marrow using melting curve analysis

Although PCR using the UTR primers and visualisation by gel electrophoresis enables the simultaneous detection of TTV and TLMV, it does not allow differentiation between the two viruses. Two of the bone marrow samples (87 and 23) had a considerably higher titre of virus present than the other four samples and it was of interest to determine if these samples were infected with TTV or TLMV of had mixed TTV/TLMV infection. To investigate this, these samples were analysed by real time PCR using Roche Lightcycler to differentiate between the two Anelloviruses, TTV and TLMV.

The primary product from the serial dilutions used to determine the endpoint of these samples was used as a template for a secondary PCR reaction on the Roche Lightcycler with the UTR primers CVOS/CVIA and SYBR green fluorescent dye. The melting curve profiles of these samples are shown in Figure 4.2.3 and Figure 4.2.4.

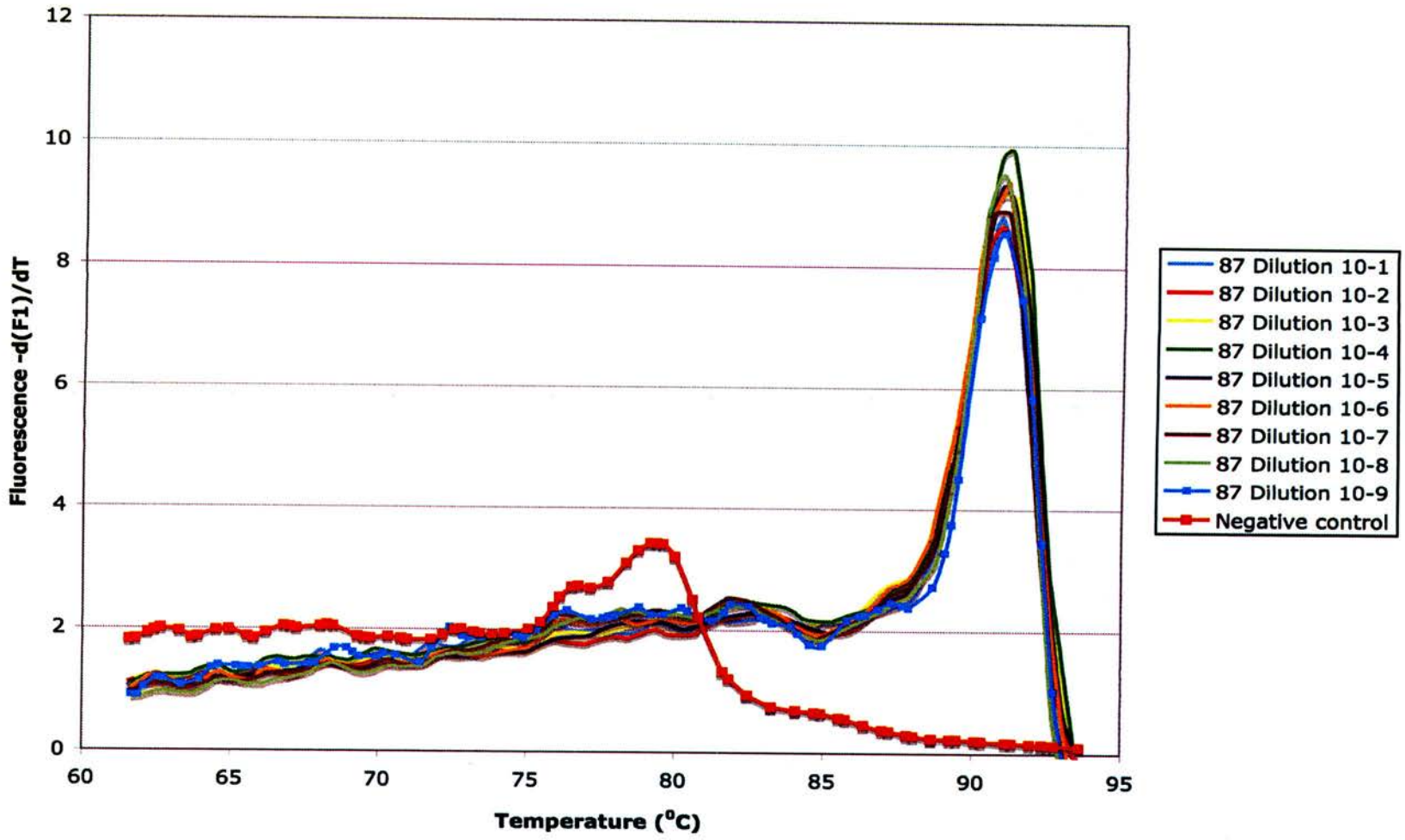

Figure 4.2.3 – Melting curve profile of ten fold serial dilution of DNA from bone marrow sample 87 amplified using UTR primers and analysed on the Roche Lightcycler.

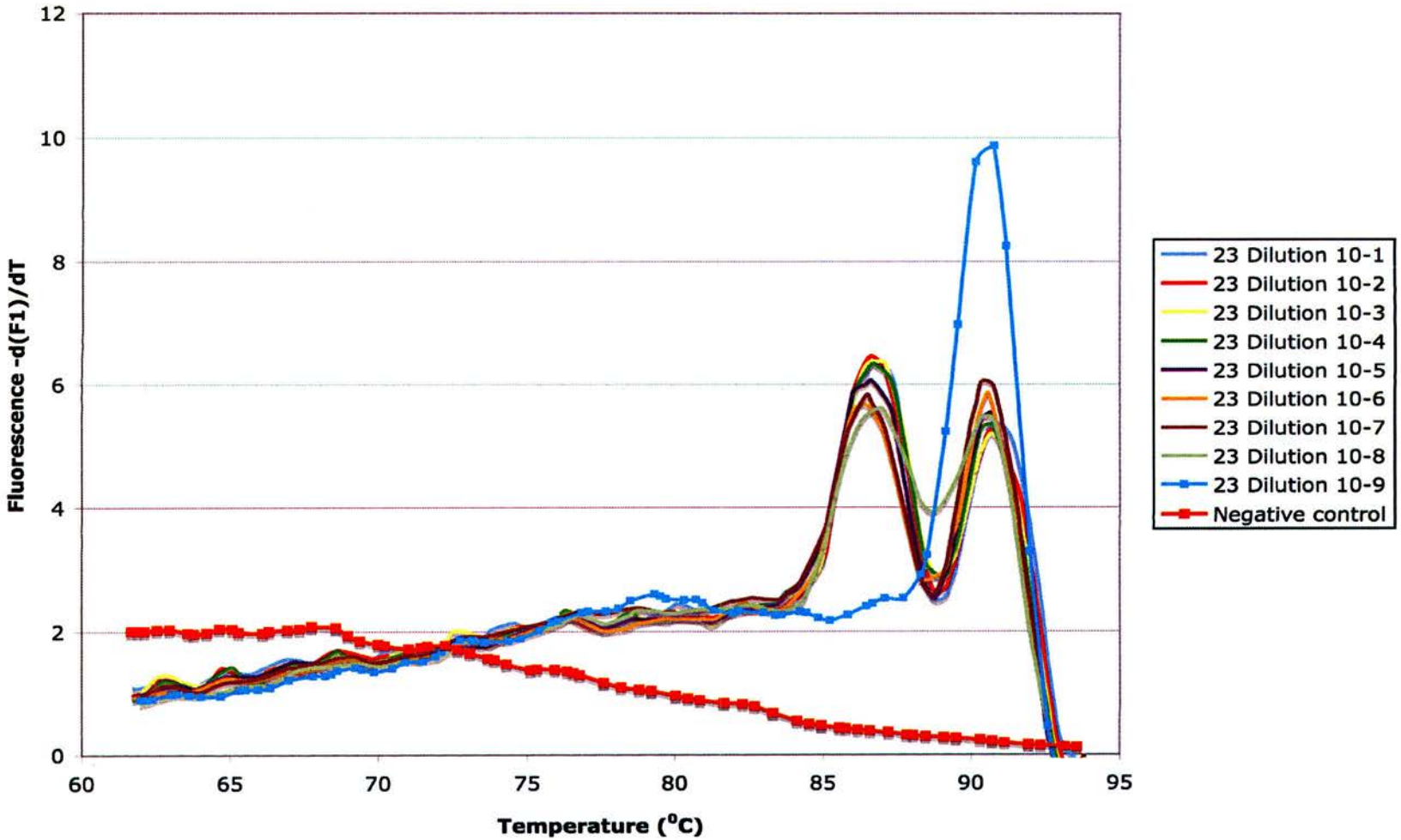

Figure 4.2.4 – Melting curve profile of ten fold serial dilution of DNA from bone marrow sample 23 amplified using UTR primers and analysed on the Roche Lightcycler.

The Lightcycler melting curve profiles show that sample 87 was infected only with TTV. Sample 23 was infected with both TTV and TLMV however the final dilution contained only TTV indicating that there was a higher titre of TTV than TLMV in that bone marrow sample.

4.2.1.2 TTV/TLMV titres in HIV negative bone marrow

Given the variable titre of Anelloviruses in the HIV positive bone marrow samples, HIV negative bone marrow samples were acquired to act as a comparison. Seven bone marrow aspirate samples from patients (five male, one female, one unknown, mean age 64.8 years, range 25-81 years) who were being investigated for haematological abnormalities were obtained from Dr Christopher Ludlum, Clinical Director of Haematology, Royal Infirmary, 51 Little France Crescent, Edinburgh, EH16 4SA. These aspirates came in the form of whole blood with bone marrow fragments suspended within it. The bone marrow fragments were separated from the whole blood sample by centrifugation, collected with a pastette and washed several times with PBS. Plasma was also collected from each whole blood sample.

Nucleic acids were isolated from the bone marrow using the phenol chloroform extraction method and the concentration quantified using a spectrophotometer. DNA from the bone marrow samples was diluted and 0.1 μg DNA added to the first dilution of the PCR followed by a further seven ten fold dilutions to endeavour to quantify the relative titre of the sample using limiting dilution PCR. A hemi-nested PCR was carried out with the UTR primers (Figure 4.2.5 and Figure 4.2.6).

Plasma corresponding to each bone marrow aspirate sample was extracted using the Qiagen QIAamp minelute virus spin kit and the viral titres estimated using limiting dilution PCR with the UTR primers (Figure 4.2.7). The last positive dilution was used to estimate the number of viral copies per ml of plasma. Sample BM7P/KG failed to give a PCR positive signal when the DNA equivalent of four μl of plasma was added to the primary PCR. The PCR for this sample was repeated with the DNA equivalent of 40 μl of plasma added and produced a band of the appropriate size when visualised on an agarose gel (not shown).

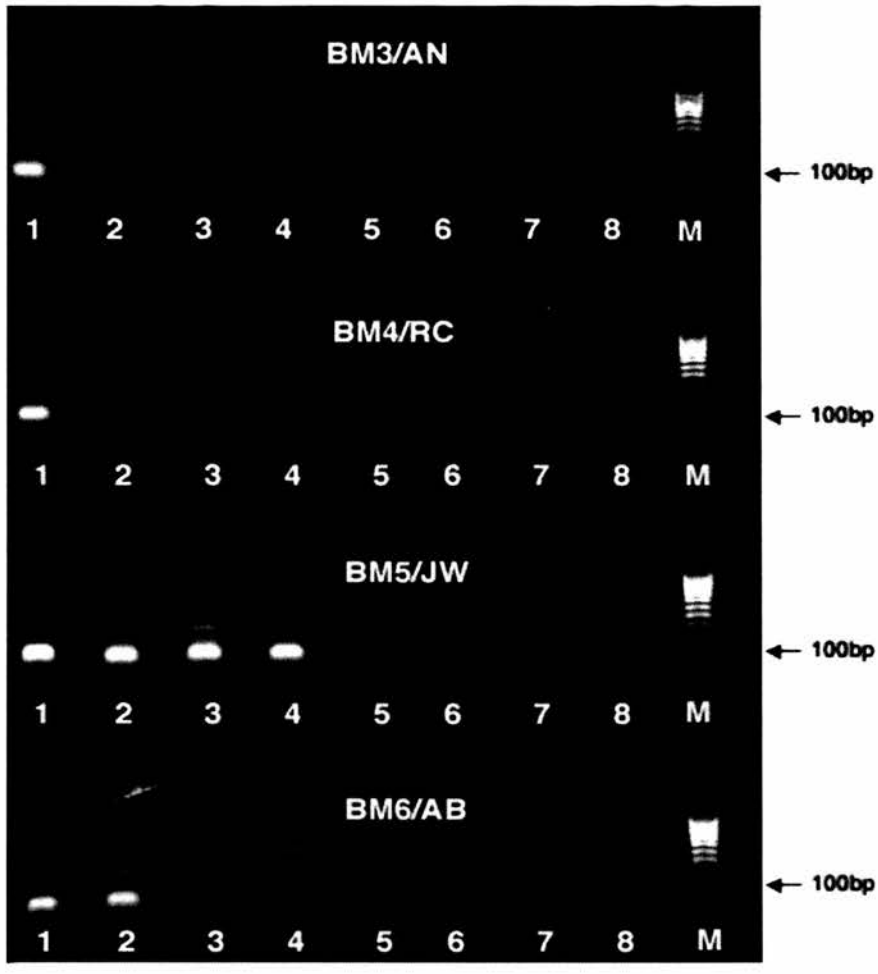

Figure 4.2.5 – PCR for TTV/TLMV on sequential dilutions of DNA extracted from bone marrow aspirates. Sample numbers are shown above each row of the agarose gel. Lanes 1-8: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: 0.1 μg DNA then 10 fold dilutions to Lane 8: $1x10^{8}$ μg DNA); Lane M: 100bp DNA ladder.

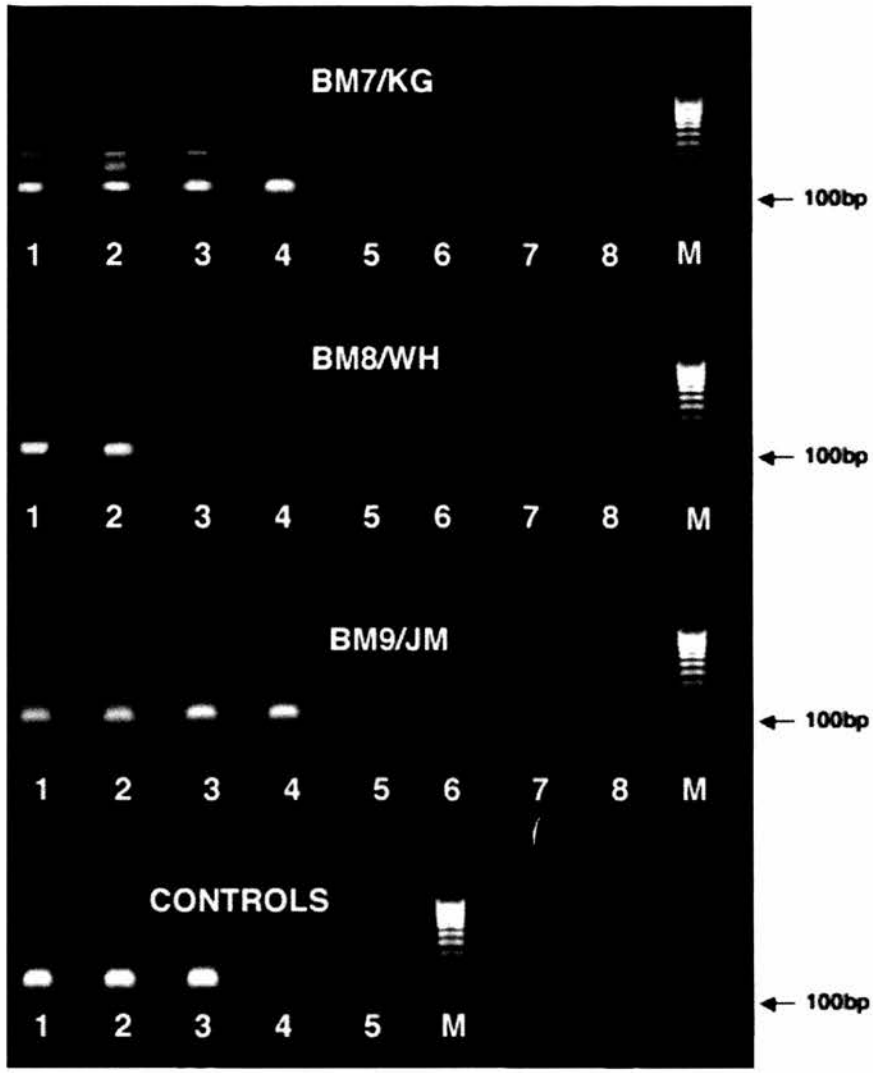

Figure 4.2.6 - PCR for TTV/TLMV on sequential dilutions of DNA extracted from bone marrow aspirates. Sample numbers are shown above each row of the agarose gel. Lanes 1-8: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: 0.1 μg DNA then 10 fold dilutions to Lane 8: $1x10^8$ μg DNA); Lane M: 100bp DNA ladder. Row 4, Lanes 1-3: Positive controls (minipools); Lanes 4&5: Negative controls (water).

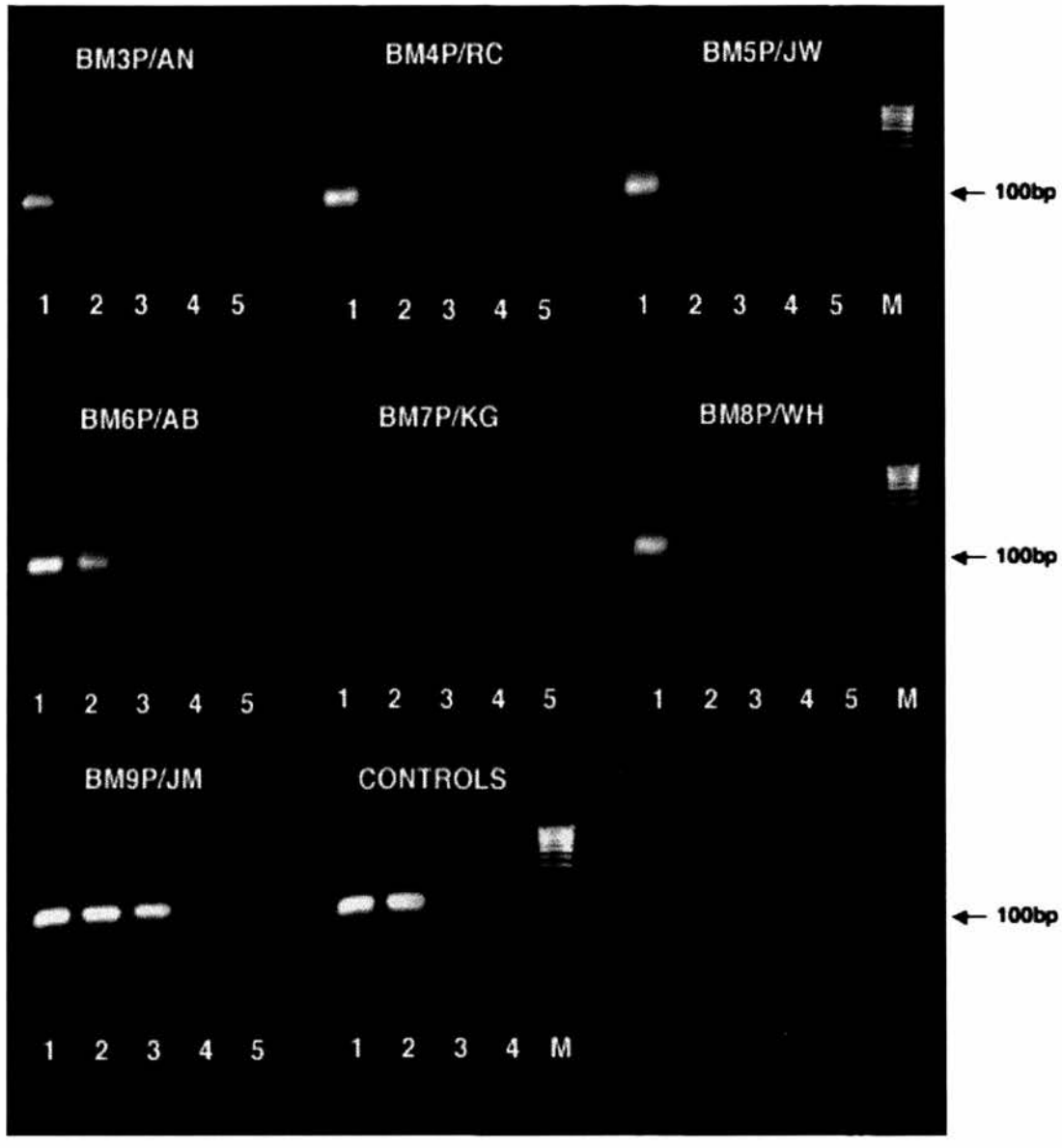

Figure 4.2.7 - PCR for TTV/TLMV on sequential dilutions of DNA extracted from plasma. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: amount of DNA equivalent to 4 µl plasma then 10 fold dilutions to Lane 5: amount of DNA equivalent to $4x10^5$ µl plasma); Lane M: 100bp DNA ladder. Controls, Lanes 1&2: Positive controls (minipools); Lanes 3&4: Negative controls (water).

The number of viral copies per bone marrow cell (mean 0.028 copies per cell, median 0.001 copies per cell) and per ml of plasma (mean 4075 copies per ml, median 250 copies per ml) were estimated (Table 4.2.2) based on the last PCR positive dilution visible on the agarose gels shown in Figure 4.2.5-Figure 4.2.7. As the graph in Figure 4.2.8 shows, there

does not appear to be any relationship between the titre of virus in bone marrow and the concentration of virus in one ml of plasma.

Sample	Age (years)	Sex (Male/Female)	Virus copies per cell – bone marrow (copies)	Virus copies per ml of plasma (copies)
BM3/AN	N/D	N/D	0.0000658	250
BM4/RC	N/D	M	0.0000658	250
BM5/JW	75	M	0.0658	250
BM6/AB	81	M	0.000658	2500
BM7/KG	25	F	0.658	25
BM8/WH	81	M	0.000658	250
BM9/JM	62	M	0.685	25000

Table 4.2.2 – Estimation of the number of copies of TTV/TLMV per bone marrow cell and number of copies of TTV/TLMV per ml of plasma from HIV negative bone marrow aspirate samples. Semi-quantitation carried out by limiting dilution PCR using UTR primers. N/D indicates no data were available. Virus copies per cell are shown to three significant figures.

4.2.1.3 Comparison of TTV/TLMV titres in HIV positive and negative bone marrow samples

Compared with the HIV positive bone marrow, the HIV negative bone marrow samples appear to contain considerably less TTV/TLMV. All of the HIV negative bone marrow samples contain less than one copy of virus per cell, ranging from one copy in 1.45 cells to one copy in 10000 cells.

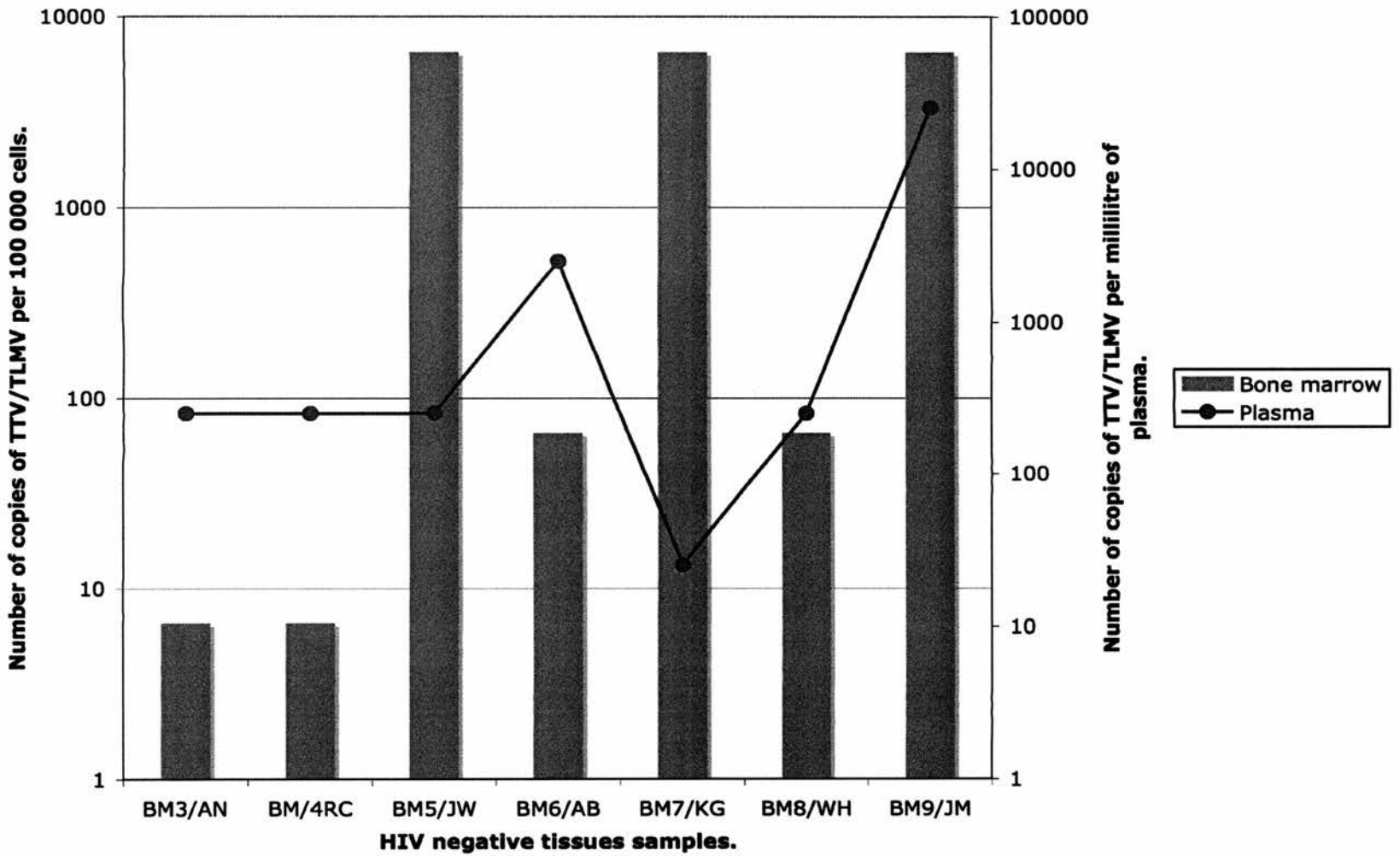

Figure 4.2.8 - Graph showing relationship between titre of virus in bone marrow and plasma from HIV negative samples.

To investigate the possibility that HIV status might influence the titre of TTV/TLMV in bone marrow, the data obtained from the quantification of the viruses in HIV positive (Table 4.2.1) and HIV negative (Table 4.2.2) bone marrow were compared (Figure 4.2.9). Statistical analysis was carried out using the Mann-Whitney U test with the null hypothesis (H_0) that there is no difference between the titre of virus in HIV positive and HIV negative bone marrow. This test produced a p-value of 0.084 which means that although the null hypothesis can not be rejected in this instance, and a significantly higher titre of TTV/TLMV in HIV positive bone marrow than in HIV negative cannot be conclusively proved, there does seem to be a trend towards this which should be investigated further using an increased sample size.

4.2.2 TTV/TLMV titres in paired HIV negative and HIV positive bone marrow and spleen samples

4.2.2.1 TTV/TLMV titres in paired HIV positive bone marrow and spleen samples

Previous experiments suggested that bone marrow from HIV positive individuals may have a higher titre of TTV/TLMV than bone marrow extracted from HIV negative individuals. In order to investigate further the titre of TTV/TLMV in HIV positive tissues, titres of Anelloviruses in pairs of tissue samples were investigated. Nineteen post mortem, HIV positive, paired bone marrow and spleen samples (15 male, four female, mean age 31.5 years, range 20-60 years) were obtained from Professor Jeanne Bell and kept at -40°C until required. Fourteen of the 19 patients (13 male, one female, median age 32 years) the cause of death was AIDS related while the cause of death in the remaining five (three male, two female, median age 30 years) was unrelated to their HIV status. DNA was extracted from these samples using the Roche High Pure PCR template kit and the DNA concentration determined using a spectrophotometer. A standardised DNA concentration was utilised as the starting point for limiting dilution PCR with UTR primers (a representative agarose gel is shown in Figure 4.2.10). The last PCR positive dilution was assumed to contain at least one copy of the virus of interest and used to estimate the number of virus copies per cell (Table 4.2.3).

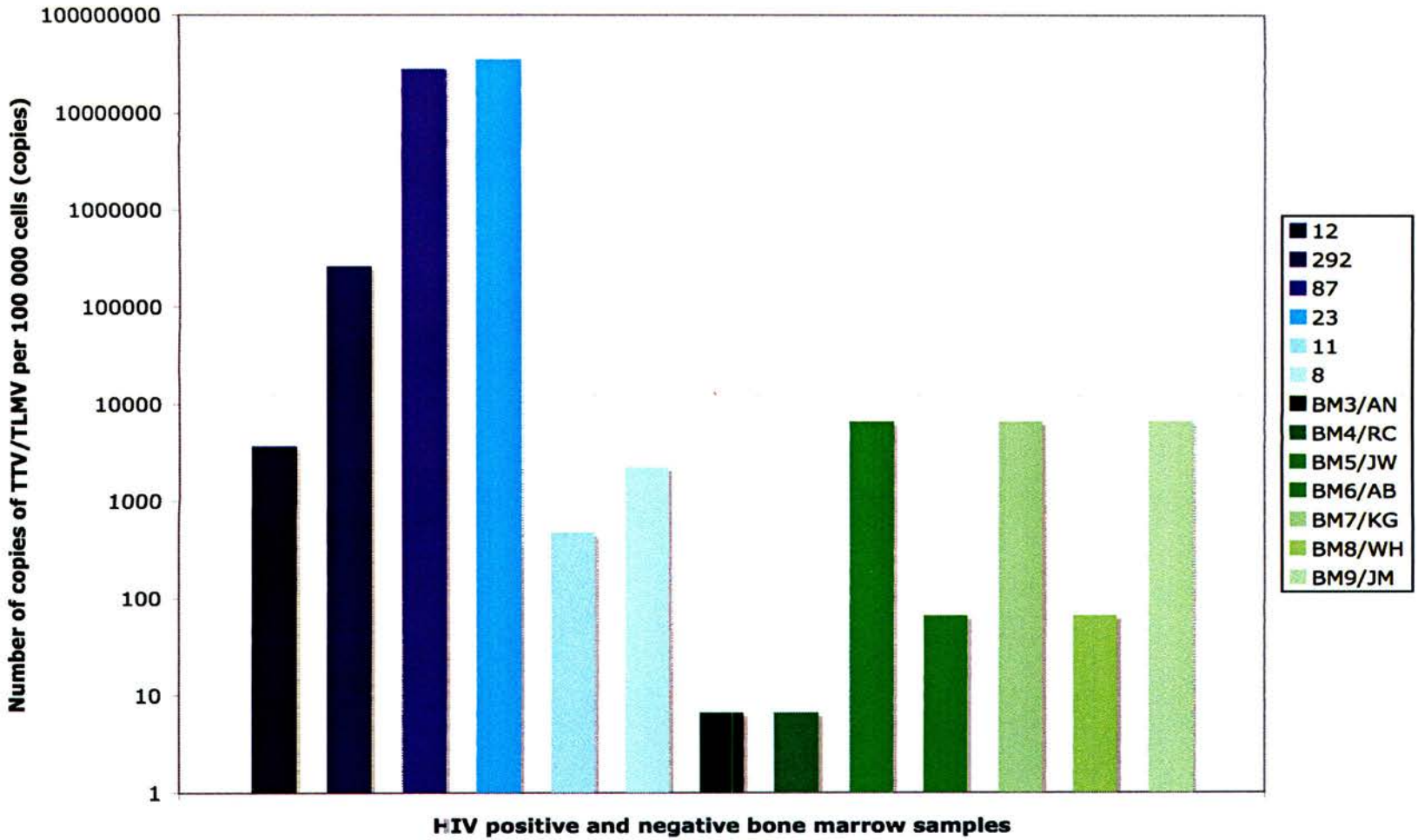

Figure 4.2.9 – Number of copies of TTV/TLMV in 100 000 cells in HIV positive (blue) and HIV negative (green) bone marrow.

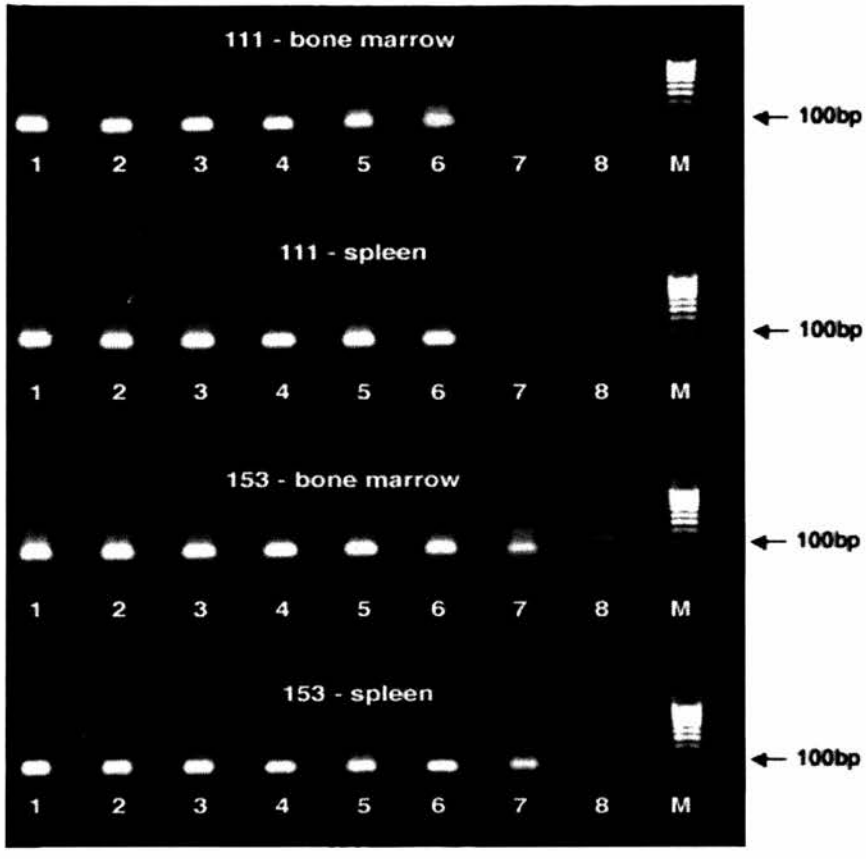

Figure 4.2.10 - PCR for TTV/TLMV on sequential dilutions of DNA extracted from paired HIV positive bone marrow and spleen samples. Sample numbers are shown above each row of the agarose gel. Lanes 1-8: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: 0.1 μg DNA then 10 fold dilutions to Lane 8: $1x10^{8}$ μg DNA); Lane M: 100bp DNA ladder.

As Table 4.2.3 shows, all of the bone marrow and spleen samples were positive for TTV/TLMV (bone marrow: mean 129.89 copies per cell, median 65.79 copies per cell; spleen: mean 63.13 copies per cell, median 6.58 copies per cell). The Mann-Whitney test was used to analyse the difference in titre of virus between bone marrow and spleen samples using the null hypothesis was there was no difference. The p-value was 0.36 so overall there was no significant difference between the titres of TTV/TLMV in these tissues.

Of the nineteen pairs of tissue samples: six pairs (31.6%) had the same titre for both tissue types, four pairs (21.1%) had one log more virus in spleen than in bone marrow, seven pairs (36.8%) had one log more TTV/TLMV in bone marrow than in spleen and two (10.5%) pairs had three logs more virus in bone marrow than in spleen.

Table 4.2.3 - Estimation of the number of copies of TTV/TLMV per bone marrow and spleen cell of HIV positive, post mortem tissue using limiting dilution PCR with primers situated in the UTR region of the genome. Individuals who died of an AIDS related illness are shown in red and individuals whose cause of death was unrelated to their HIV status are shown in orange. * indicates samples found to be co-infected with hepatitis C virus. CD4+ T cell counts provided by Jeanne Bell. N/D indicates that no data were available. The number of viral copies per cell is shown to three significant figures.

Sample	Age(year)	Sex (Male/Female)	Tissue (bone marrow/spleen)	Virus copies per cell (copies)	CD4+ T cell count (cells/mm)
111	60	M	Bone marrow	6.58	7
			Spleen	658	
153*	N/D	M	Bone marrow	65.8	1
			Spleen	65.8	
102	N/D	M	Bone marrow	65.8	3
			Spleen	65.8	
240	49	M	Bone marrow	65.8	0
			Spleen	65.8	
180	31	M	Bone marrow	6.58	40
			Spleen	65.8	
99	33	M	Bone marrow	65.8	0
			Spleen	658	
145	44	M	Bone marrow	6.58	N/D
			Spleen	6.58	
209	20	M	Bone marrow	658	N/D
			Spleen	65.8	

173*	30	M	Bone marrow	6.58	170
			Spleen	0.658	
181*	27	M	Bone marrow	658	7
			Spleen	65.8	
184	25	M	Bone marrow	0.00658	N/D
			Spleen	0.000658	
135	59	M	Bone marrow	65.8	N/D
			Spleen	0.0658	
117	28	F	Bone marrow	658	N/D
			Spleen	0.658	
155*	45	M	Bone marrow	6.58	1
			Spleen	0.658	
188*	28	M	Bone marrow	6.58	230
			Spleen	65.8	
222	31	F	Bone marrow	0.0658	247
			Spleen	0.0658	
156*	32	F	Bone marrow	65.8	200
			Spleen	6.58	
194	28	M	Bone marrow	0.658	200
			Spleen	0.0658	
224	29	F	Bone marrow	0.000658	245
			Spleen	0.00658	

Using the Wilcoxon signed ranks test the differences between bone marrow and spleen samples from the same individual were analysed (p=0.22), however again the differences were not shown to be significant. Differences between the titre of TTV/TLMV in bone marrow and spleen for individuals within the HIV positive and AIDS groups were also not found to be significant (AIDS p=0.24, HIV positive p=0.68).

Analysis to compare the titre of TTV/TLMV in bone marrow between the two groups showed significantly higher titre of virus in the AIDS cohort compared with the and HIV positive group (p=0.006). Significantly higher titres were also seen in the AIDS cohort when the TTV/TTMV was quantified in spleen (AIDS vs. HIV positive: p=0.022). This supports the role of the immune system in regulating TTV/TTMV titre although the exact mechanism of this remains unclear.

Where available, the CD4+ T cell counts were provided by Jeanne Bell to give an indication of the level of immunosuppression of the individual so any relationship between the immune status and virus titre could investigated further. To this end, Spearman rank-order correlations were used to test for correlations between the titre of TTV/TLMV in bone marrow and spleen and the CD4+ T cell count for the same individual. This test gave a p-value of 0.007 for bone marrow and p=0.008 for spleen indicating a inverse correlation between the number of copies of the virus per cell and CD4+ T cell count. Thus it appears that as the CD4+ T cell count of an individual drops as they progress towards AIDS, the titre of TTV/TLMV in both bone marrow and spleen increases significantly.

4.2.2.1.1 Differentiation of TTV and TLMV in HIV positive paired bone marrow and spleen samples on Roche Lightcycler

Again, it was deemed appropriate to use real-time PCR analysis on the Roche Lightcycler to characterise the nature of the Anelloviruses present. Primary product from 4.2.2.1 was used as a template and following amplification, the secondary products were heated to 95°C to allow differentiation of TTV and TLMV by melting temperature (Figure 4.2.11 and Figure 4.2.12).

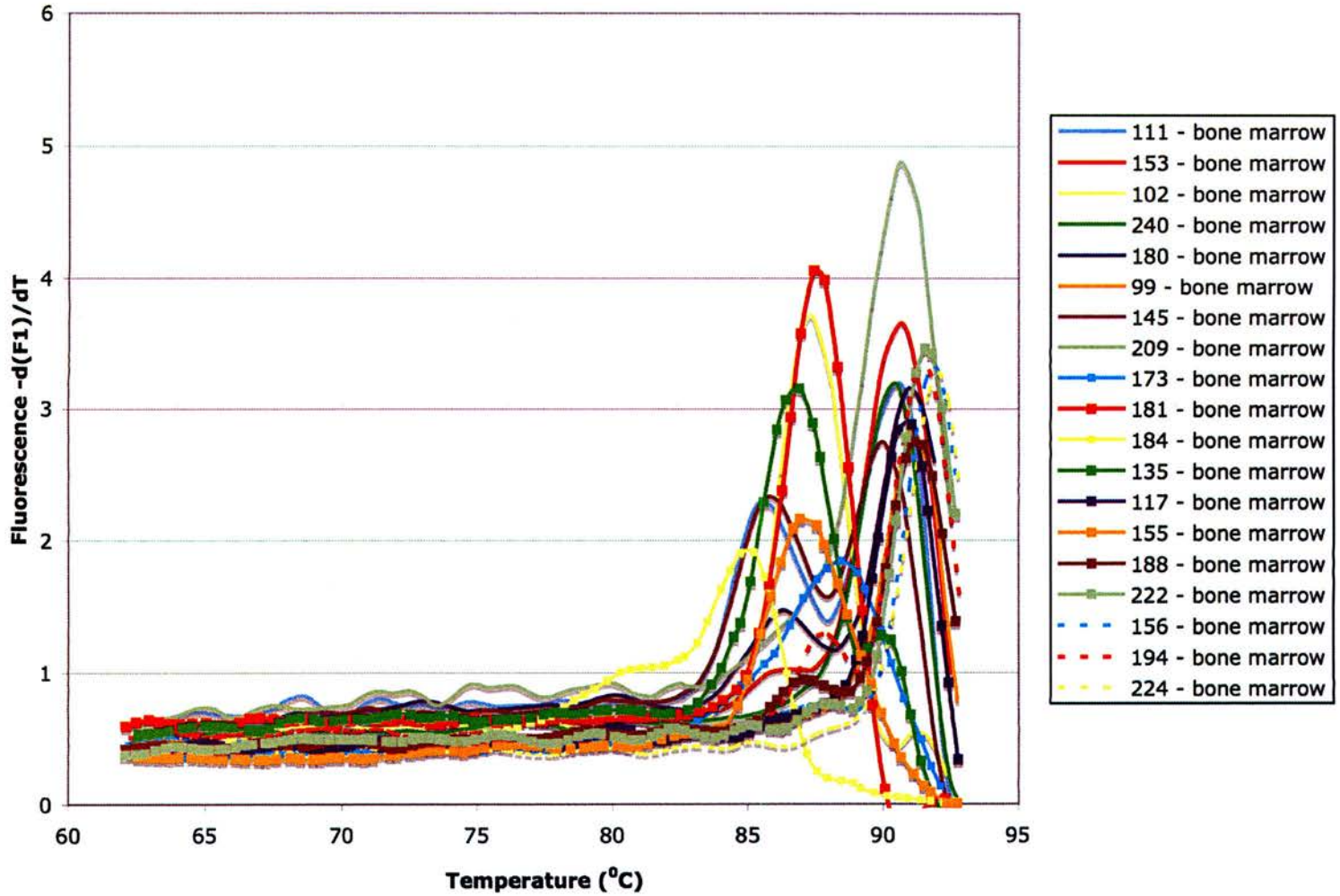

Figure 4.2.11 – Melting peak profiles of DNA extracted from HIV positive bone marrow samples and amplified using primers from the UTR region of the TTV and TLMV genomes.

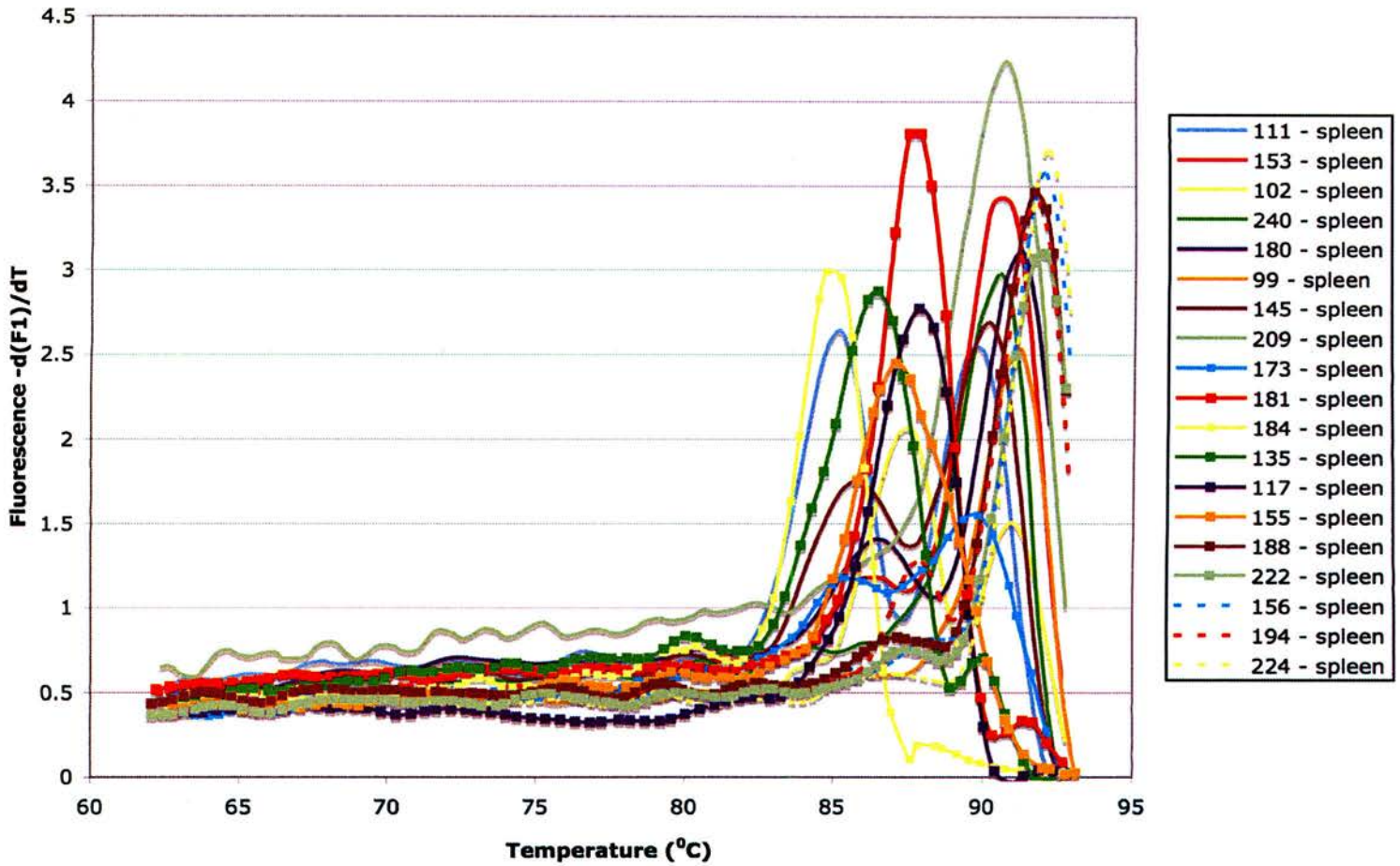

Figure 4.2.12 – Melting peak profiles of DNA extracted from HIV positive spleen samples and amplified using primers from the UTR region of the TTV and TLMV genomes.

As Table 4.2.4 shown, the HIV positive samples show a range of single TTV and TLMV infection and dual infection with both Anelloviruses. Of the nineteen bone marrow samples, eight (42.1%) were positive for TTV, five were positive for TLMV (26.3%) and six (31.6%) were positive for both TTV and TLMV. Six (31.6%) of the nineteen spleen samples were positive for TTV, four (21.1%) for TLMV and nine (50%) for both viruses.

	Bone marrow		Spleen	
Sample	TLMV	TTV	TLMV	TTV
111	+	+	+	+
153	-	+	+	+
102	+	-	+	+
240	-	+	-	+
180	+	+	+	+
99	-	+	-	+
145	+	+	+	+
209	-	+	-	+
173	+	-	+	+
181	+	-	+	-
184	+	-	+	-
135	+	+	+	+
117	-	+	+	-
155	+	-	+	-
188	+	+	+	+
222	-	+	-	+
156	-	+	-	+
194	+	+	+	+
224	-	+	-	+

Table 4.2.4 – Frequency of TLMV (shown in green), TTV (shown in blue) and mixed TTV/TLMV infection (shown in red) in paired HIV positive bone marrow and spleen samples.

4.2.2.1.2 Hepatitis C infection in HIV positive bone marrow and spleen samples

It has been well documented that viral co-infections can change the dynamics of an infection, for example the Flavivirus GBV-C has a positive impact on disease progression in HIV infected patients [Northfield, 2005]. HIV and HCV share transmission routes and, as such, up to 30% of those infected with HIV also have a chronic hepatitis C infection, a figure which increases when applied to HIV positive intravenous drug users [Mohsen, 2002]. In cases of co-infection, it appears HIV can increase the disease progression of hepatitis C and in vitro studies have shown the HCV replication rate can be increased by as much as eight times in an individual who is also infected with HIV [Northfield, 2005]. The effect of HCV/HIV co-infections on the titre of TTV/TLMV has yet to be ascertained, therefore it was of interest to investigate the HCV status of the HIV samples used in 4.2.2.1.

Nineteen paired, HIV positive, bone marrow and spleen samples were tested for the presence of Hepatitis C virus to investigate the effect of HCV/HIV co-infections on the titre of TTV/TLMV present in the tissues. Nucleic acid from each sample was subjected to a nested PCR and visualised on the Roche Lightcycler (Figure 4.2.13). Unlike TTV and TLMV, the HCV genome is sufficiently conserved to allow the use of sequence specific probes for detection on the Lightcycler instead of the non-specific SYBR green used for the detection of Anelloviruses. Of the 38 samples tested, six bone marrow samples (153, 173, 181, 155, 188, and 156) and one spleen sample (156) were positive for the presence of HCV.

The Mann-Whitney U test was used to investigate any difference in titre between samples co-infected with HCV and HIV compared with those infected with HIV alone. The null hypothesis was that there was no difference between the two groups of samples. The p-value produced using this test was 0.69 for bone marrow and 0.52 for spleen indicating that co-infections of HCV and HIV did not significantly alter the titre of TTV/TLMV in the bone marrow and spleen samples.

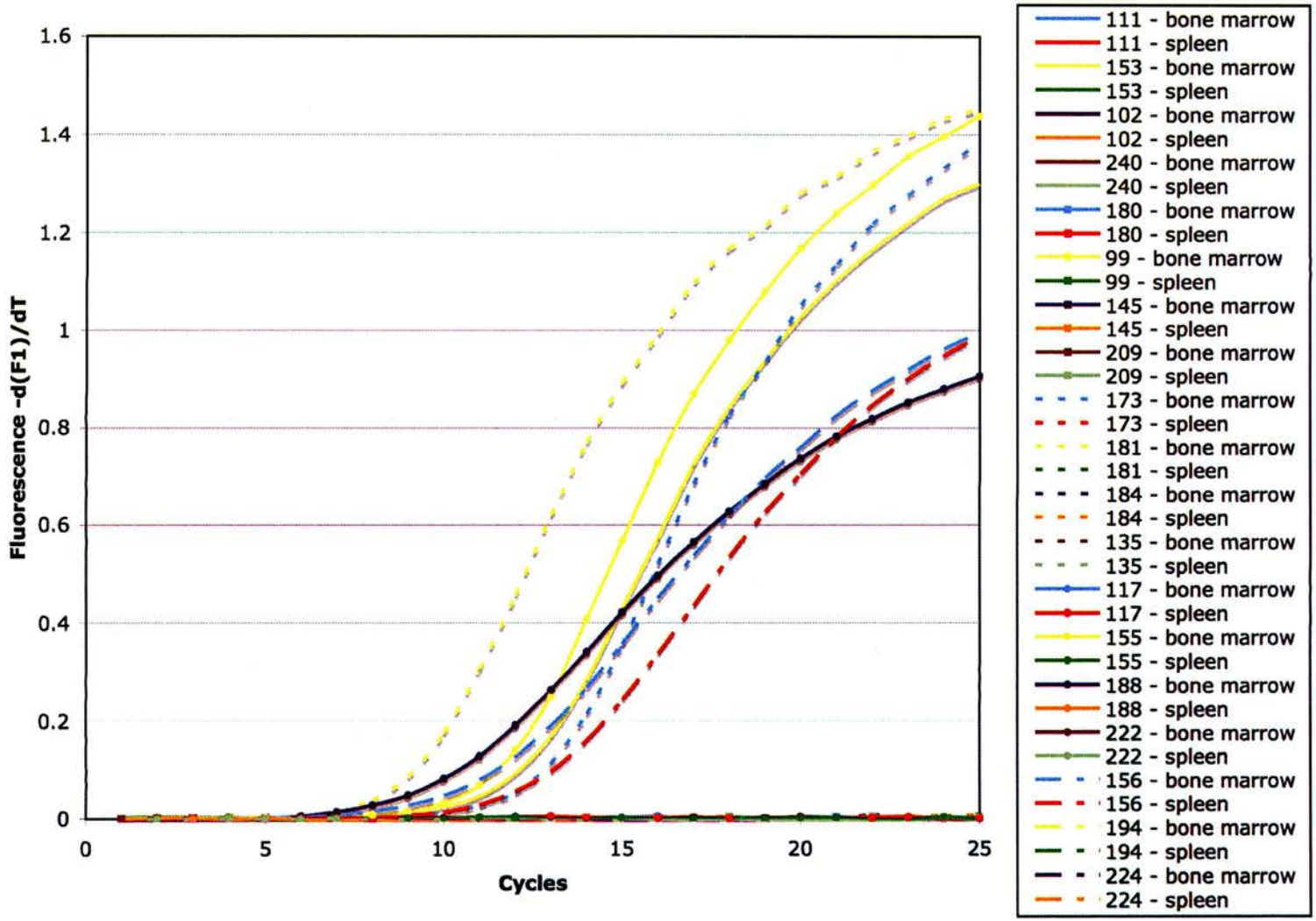

Figure 4.2.13 – Amplification of HCV from HIV positive paired bone marrow and spleen samples on the Roche Lightcycler.

4.2.3 TTV/TLMV titres in paired HIV negative bone marrow and spleen samples

In order to compare the results obtained in 4.2.2.1 with a group who were not immunocompromised, seven HIV negative paired bone marrow and spleen samples (two male, two female, three unknown; mean age 78.6 years, range 69 to 84 years) were obtained from Jeanne Bell and stored at -40°C until required. DNA was extracted from each of them using the Roche High Pure PCR template kit and the concentration of DNA was determined using a spectrophotometer. Limiting dilution PCR was used to estimate the number of virus copies present. A representative agarose gel is shown in Figure 4.2.14. The last positive dilution from each of the samples was used to calculate an approximate number of copies of TTV/TLMV per cell (Table 4.2.5).

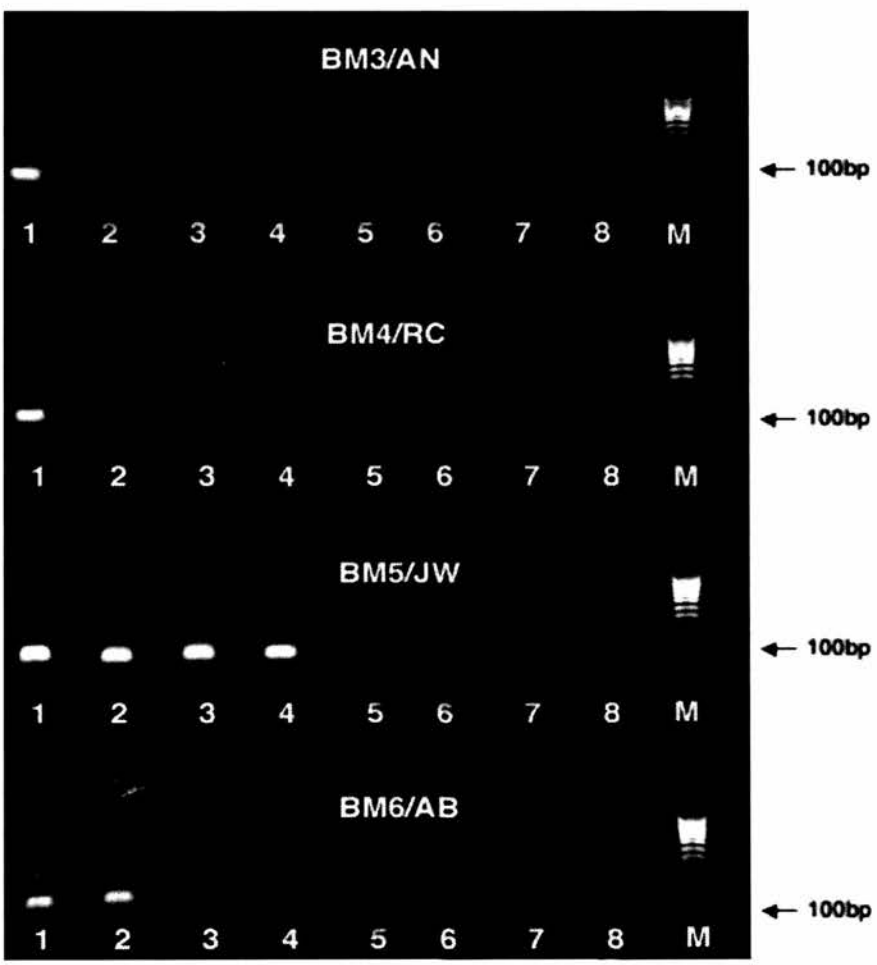

Figure 4.2.14 - PCR for TTV/TLMV on sequential dilutions of DNA extracted from paired HIV negative bone marrow and spleen samples. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: 0.1 μg DNA then 10 fold dilutions to Lane 5: $1x10^5$ μg DNA); Lane M: 100bp DNA ladder.

Sample	Age (years)	Sex (Male/Female)	Tissue (bone marrow/spleen)	Virus copies per cell (copies)
A	83	F	Bone marrow	0.0658
			Spleen	0.0058
DFN	N/D	M	Bone marrow	0.0000658
			Spleen	0.00658
B	84	F	Bone marrow	0.00658
			Spleen	0.0658
C	69	M	Bone marrow	0.0658
			Spleen	0.000658
F395	N/D	N/D	Bone marrow	0.0658
			Spleen	0.00658
F396	N/D	N/D	Bone marrow	0.00658
			Spleen	0.0658
F407	N/D	N/D	Bone marrow	0.0658
			Spleen	0.0658

Table 4.2.5 - Estimation of the number of copies of TTV/TLMV per bone marrow and spleen cell of HIV negative, post mortem tissue using limiting dilution PCR with primers situated in the UTR region of the genome. N/D indicates that no data were available. Values for number of virus copies per cell are shown to three significant figures.

As the table shows, all of the bone marrow and spleen samples were positive for Anelloviruses (bone marrow: median 0.039 copies per cell, mean 0.066 copies per cell; spleen: median 0.031 copies per cell, median 0.006 copies per cell). Of the seven pairs of samples, two pairs had the same titre of TTV/TLMV in both the bone marrow and spleen.

The other five pairs had differences between the pairs of tissues with a maximum difference in titre of two logs.

The Mann-Whitney U test, used to compare the number of copies of TTV/TLMV per cell in bone marrow and spleen, gave a p-value of 0.65. Any significant differences between the titre of virus in the bone marrow and spleen on the same individual were investigated using the Wilcoxon signed ranks test, which gave a p-value of 0.59. These statistics show there is no significant difference in Anellovirus titre between the bone marrow and spleen samples as a group and on an individual level.

The Mann-Whitney U test was used again, this time to compare the titre of TTV/TTMV in bone marrow between the AIDS and HIV positive samples from section 4.2.2.1 with bone marrow from HIV negative patients. This analysis showed significantly higher titres of virus in the AIDS cohort compared with both the HIV positive group ($p=0.006$) and the HIV negative group ($p<0.001$). Significantly higher titres were also seen in the AIDS cohort when the TTV/TLMV was quantified in spleen (AIDS vs. HIV positive: $p=0.022$, AIDS vs. HIV negative: $p<0.001$). The increased titre in AIDS patients who are severely immunocompromised suggests the changes associated with this immunodeficiency in some way affect the dynamics of Anellovirus infection.

4.2.3.1 Differentiation of TTV and TLMV in HIV negative paired bone marrow and spleen samples on Roche Lightcycler

The first dilution of primary product from each of the seven HIV negative paired bone marrow and spleen samples were run on the Lightcycler to differentiate between TTV and TLMV in each of the tissue samples. Primers CVOS and CVIA were used in conjunction with SYBR green fluorescent dye to amplify a product of approximately 100bp that was then subjected to melting curve analysis to determine the Tm of each of the samples (Figure 4.2.15).

Table 4.2.6 shows the distribution of TTV and TLMV in each of the samples. Of the seven bone marrow samples, two (28.6%) were positive for TTV and five (71.4%) for both TTV and TLMV. Two (28.%) of the spleen samples had a Tm consistent with the presence of TTV, one (14.3%) was TLMV positive and four (57.1%) had mixed TTV/TLMV infection.

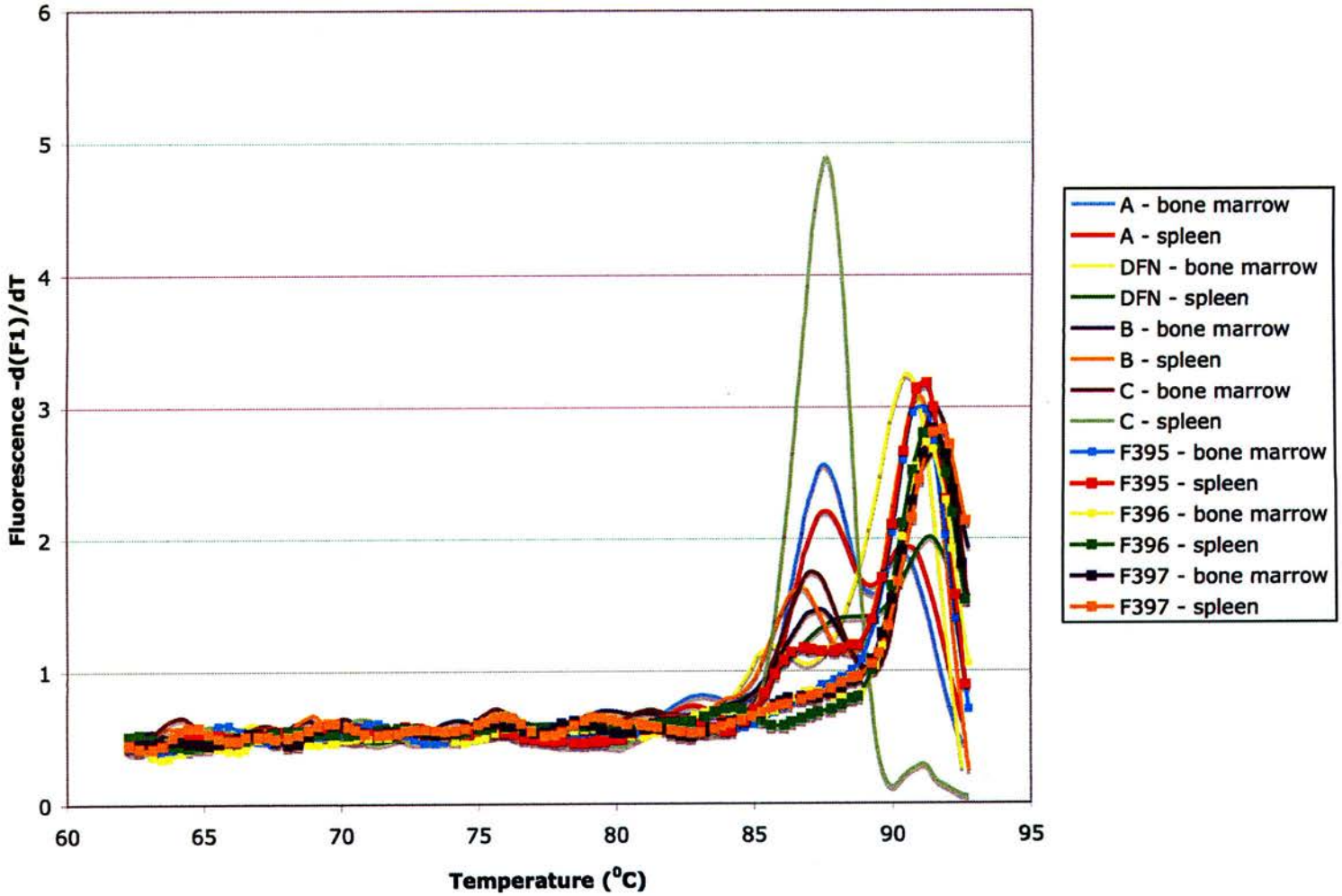

Figure 4.2.15 – Melting curve profile of HIV negative paired bone marrow and spleen samples amplified with UTR primers and analysed on the Roche Lightcycler using SYBR green fluorescent dye.

	Bone marrow		Spleen	
Sample	TLMV	TTV	TLMV	TTV
A	+	+	+	+
DFN	+	+	+	+
B	+	+	+	+
C	+	+	+	-
F395	-	+	+	+
F396	+	+	-	+
F407	-	+	-	+

Table 4.2.6 – Frequency of TLMV (shown in green), TTV (shown in blue) and mixed TTV/TLMV infection (shown in red) in paired HIV negative bone marrow and spleen samples.

4.2.4 TTV/TLMV titres in HIV positive tissue samples

Okamoto studied the titre of TTV found in the tissues of three HIV negative individuals and discovered that although titres appeared to be higher in bone marrow, lung, spleen, and liver than in other tissues (lymph node, muscle, thyroid gland, pancreas and kidney) the viral load varied between individuals [Okamoto et al., 2001a]. Previous experiments within this study showed similar titres of Anelloviruses in bone marrow and spleen samples. To investigate the titre of TTV/TLMV in a wider range of tissues from an HIV positive individual, four further tissue samples were obtained from individual 224 (Table 4.2.3), a female aged 29 years who died pre-AIDS of a dipipanone over dose.

Nucleic acid was extracted from bone marrow, spleen, left cerebellum, lymph node, thoracic spinal cord and liver using the Roche High Pure PCR Template kit. The DNA concentration was analysed before limiting dilution PCR analysis was carried out (Figure 4.2.16 and Figure 4.2.17). The last PCR positive dilution was used to estimate the number of viral copies per cell.

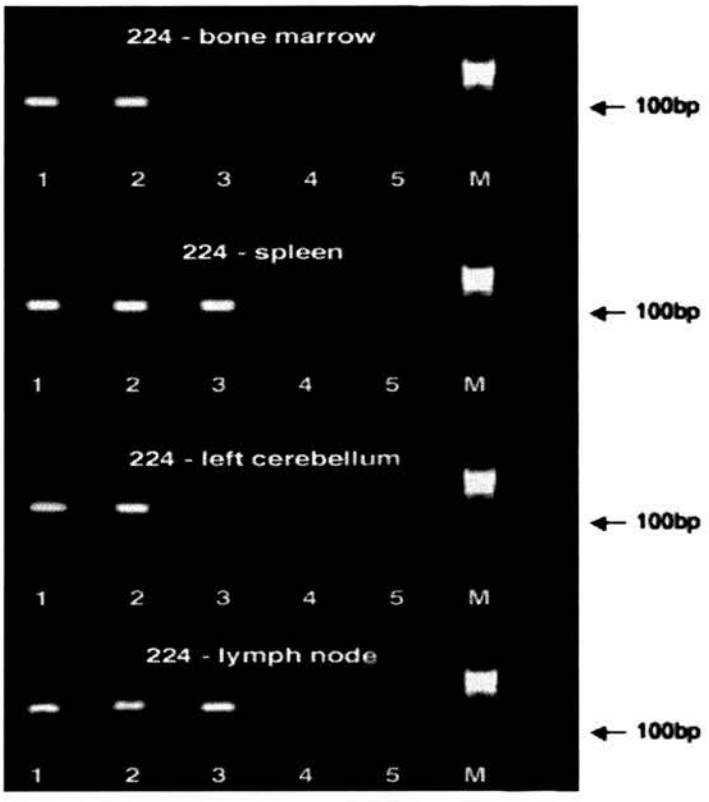

Figure 4.2.16 - PCR for TTV/TLMV on sequential dilutions of DNA extracted from bone marrow, spleen, cerebellum and lymph node samples. Tissue types are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: 0.1 μg DNA then 10 fold dilutions to Lane 5: $1x10^5$ μg DNA); Lane M: 100bp DNA ladder.

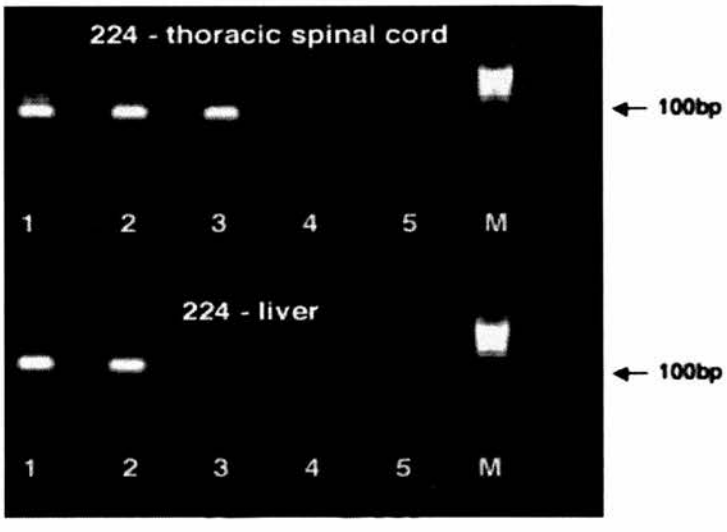

Figure 4.2.17 - PCR for TTV/TLMV on sequential dilutions of DNA extracted from thoracic spinal cord and liver samples. Tissue types are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: 0.1 μg DNA then 10 fold dilutions to Lane 5: $1x10^5$ μg DNA); Lane M: 100bp DNA ladder.

Bone marrow, cerebellum and liver had roughly one viral copy per 1500 cells while spleen, lymph node and thoracic spinal cord had one viral copy per 150 cells. A graph of the number of copies of TTV/TLMV per 100 000 cells for all of the tissue types is shown in Figure 4.2.18.

4.2.4.1 Differentiation of TTV and TLMV from HIV positive tissue samples

To investigate the populations of TTV and TLMV present in the different tissue types, the second round of the hemi-nested PCR was repeated however, this time the amplification was carried out on the Roche Lightcycler using SYBR green as a fluorescent dye to label the amplification reaction. Differentiation of TTV and TLMV was carried out by analysing the differences in Tm between the two viruses after melting curve analysis had been carried out (Figure 4.2.19).

Analysis of the melting curves of the samples shows that tissues either contain TLMV (cerebellum), TTV (bone marrow, thoracic spinal cord, lymph node and liver) or a heterogeneous mixture of both viruses (spleen).

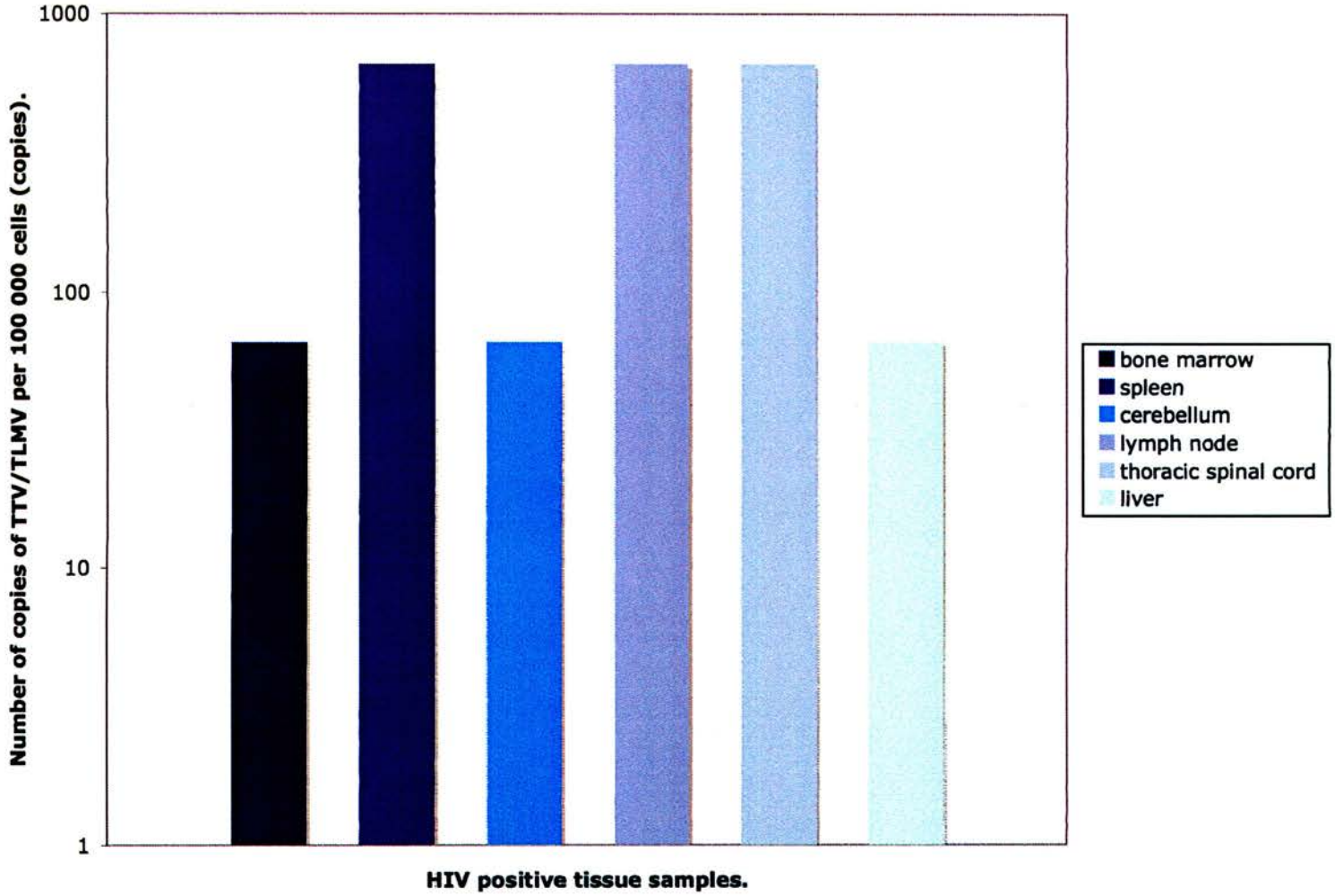

Figure 4.2.18 – Estimate of the number of copies of TTV/TLMV in 100 000 cells in tissues from HIV positive individual 224.

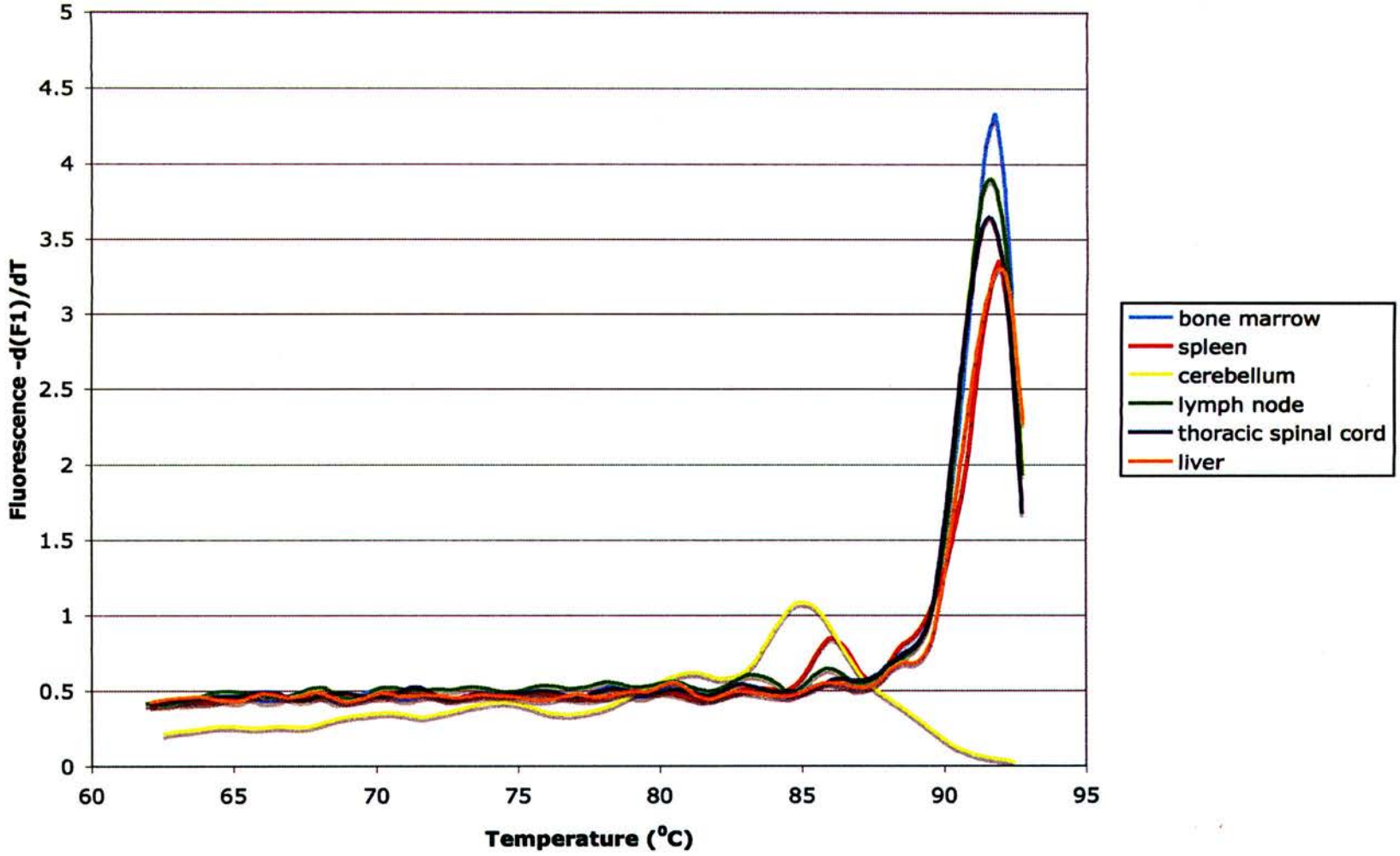

Figure 4.2.19 – Melting curve profiles from nucleic acid extracted from tissues of HIV positive individual 224, amplified using UTR primers and analysed using the Roche Lightcycler.

4.3 Discussion

The compromised immune system resulting from HIV infection is well documented, as is the risk of opportunistic infections as immunosuppression becomes more severe on progression towards AIDS. Co-infections with other viruses are common in HIV infected patients and evidence is mounting that these can result in the modification of the HIV disease processes.

TTV and TLMV are small, circular, DNA viruses which can be transmitted through parenteral, non-parenteral and faecal oral routes [Puig-Basagoiti et al., 2000; Tawara et al., 2000]. Since they share some of the same transmission routes as HIV, TTV and/or TLMV co-infections in HIV infected individuals are common. TTV was originally thought to be a candidate novel hepatitis virus and although its role in liver disease remains controversial, until its pathogenic potential is fully characterised it will remain relevant to investigate the implications in those with immune dysfunctions.

This study used a semi-quantitative method to investigate Anellovirus titre in bone marrow and spleen tissues from three cohorts: HIV positive individuals who died of AIDS related infections, HIV positive patients who died pre-AIDS and HIV negative individuals. It has been shown for the first time in this study that significantly increased viral loads are present in tissues removed from those with an AIDS defining illness compared with both the HIV positive and negative groups suggesting that the immune system does have a role of regulating TTV and TLMV viraemia. Although not thought to be significant, it is noteworthy that the sampling methods were different for the HIV positive and negative bone marrow. The HIV positive bone marrow was removed during post mortem. The HIV negative bone marrow, on the other hand, was extracted from living patients during investigation for haematological abnormalities and came in the form of aspirates.

A significant inverse association between CD4+ T lymphocyte count TTV/TLMV titre was also established, corroborating studies showing and inverse relationship between TTV viral load and level of immunosuppression [Shibayama et al., 2001; Touinssi et al., 2001]. There was no correlation between the TTV/TLMV viral load in bone marrow and spleen and the sex of the infected individual ($p>0.05$). The role of the immune system in the natural history of these viruses is further implicated by the evidence that TTV viraemia is decreased in response to interferon therapy for concomitant HCV infection [Nishizawa et al., 2000]. However, to conclude that CD4+ T cells are in some way central to the

clearance of TTV is contrary to studies suggesting that the immune reconstitution associated with high-activity antiretroviral therapy (HAART) did not lead to a decrease in TTV viraemia [Takamatsu et al., 2001].

Analogies can be made between TTV/TLMV and Chicken anemia virus, which shares genomic organisation with TTV. CAV replicates in both the bone marrow and thymus of infected chickens, causing anemia, thrombocytopenia and depletion of T lymphocytes. This is aggravated upon co-infection with a second virus. There are several facets of HIV infection which are largely unexplained, namely thrombocytopenia, neutropenia and anemia, which could be attributed to the high titre of TTV/TLMV seen in the bone marrow of HIV infected individuals. Indeed, TTV has already been implicated in thrombocytopenia and aplastic anemia [Safadi et al., 2001; Tokita et al., 2001]

Although there was no significant correlation found in this study between the TTV/TLMV titre and age, it should be noted that the median age of the individuals in both the HIV positive and the AIDS groups was lower than the HIV negative cohort. This is not thought to have influenced the increased titre of TTV/TLMV seen in the AIDS cohort. TTV has been detected in a wide range of age groups, however there is evidence to suggest that prevalence of TTV infection and virus titre increases with the increasing age of the population studied [Nagano et al., 1999; Saback et al., 1999]. If this was true of the populations studied here, it would suggest an even greater difference in titre between the AIDS cohort and negative groups.

Co-infections of HIV and HCV are very common due to the shared routes of transmission, so it was important to investigate the HCV status of the tissues analysed in this study. None of the HIV negative samples were HCV PCR positive however, six of the HIV positive samples were also infected with HCV. It should be noted that detection of Hepatitis C virus RNA in extrahepatic tissues is controversial, and although it has been detected in both bone marrow and spleen of AIDS patients, it is unknown if HCV can infect or be detected in bone marrow and spleen cells in immunocompetent individuals. It therefore cannot be confirmed that the lack of a positive PCR signal represents an individual not harbouring HCV infection. The HCV status of the individuals used in this study did not appear to alter the titre of TTV/TLMV in their tissues. It is generally accepted now that TTV infection does not affect HCV titre or reduce the efficacy of interferon treatment [Maggi et al., 2003; Nishizawa et al., 2000] however there is a body of evidence to suggest that HCV adversely affects the course of HIV infection and the

baseline CD4+ cell count is reduced in HIV/HCV co-infected patients [Northfield, 2005]. Although the evidence presented here suggests that a combination of Anelloviruses and HCV/HIV infections does not serve to increase the titre of TTV/TLMV, it does not draw any conclusions about the effect on HCV and HIV themselves.

It is hypothesised that TTV may be able to replicate in the haematological compartment and double stranded replicative intermediates have been found in the bone marrow as well as in liver in humans [Okamoto et al., 2000d; Okamoto et al., 2000e] and in the liver, bone marrow and small intestine of experimentally infected rhesus monkeys [Xiao et al., 2002]. Peripheral blood mononuclear cells (PBMCs) were thought to act as a reservoir for TTV but double stranded intermediates have not been isolated from these cells suggesting that this is not a site of replication [Okamoto et al., 2000d]. However, TTV replication has been achieved *in vitro* by stimulation of PBMCs with phytohemagglutinin, lipopolysaccharide, and interleukin-2 [Desai, 2005; Mariscal et al., 2002].

The lack of significant differences in titres of TTV/TLMV between the paired bone marrow and spleen tissues in any of the three groups investigated here suggests the spleen is also a site of replication for these viruses or the cells which are the main site of replication are able to infiltrate spleen tissue. There was also no apparent association between the titre of virus in plasma and bone marrow suggesting that the virus present in bone marrow is not just a result of circulating virus. This conclusion was also reached by Pollicino and colleagues [Pollicino et al., 2003] who found consistently lower titre of TTV in sera of samples compared with the corresponding paired tissue samples.

The use of PCR primers, which can simultaneously amplify both TTV and TLMV, and the real time PCR method of differentiating between them provides much needed data on the relationship between these viruses. These data show that like TTV, TLMV can infect and possibly replicate in bone marrow and spleen tissue both independently and in the presence of TTV. Surprisingly, the highest percentage of dual TTV and TLMV infection occurred in the bone marrow of the HIV negative cohort, which had the lowest virus titre. There was no significant association between the presence of dual TTV and TLMV infection and Anellovirus titre in either the bone marrow or spleen tissues.

It has not yet been ascertained if the increased titre of virus in immunosuppressed individuals observed here and in other publications is the result of an increase in viral replication or a decrease of immune mediated viral clearance. A high percentage (71%) of

mixed infections, sometimes with up to five isolates of TTV from different genotypes, have been noted in HIV infected patients compared with blood donors (21%) and HBV carriers (29%) [Devalle and Niel, 2004]. It is possible that people with compromised immune systems are unable to mount an immune response on exposure to new genotypes of TTV. This would support the finding that stimulation of the immune system with interferon can result in clearance or reduction of TTV viraemia [Akahane et al., 1999; Hagiwara et al., 1999; Maggi et al., 2001c].

The presence of TTV in extrahepatic tissue is the subject of a number of scientific papers [Okamoto et al., 2001a; Pollicino et al., 2003; Suzuki et al., 2001] however, since many of the samples used in this study were from HIV positive individuals it was considered of importance to further investigate presence of TTV/TLMV in a wider range of tissues. Given the increased titre of TTV/TLMV seen in the tissues taken from the AIDS cohort, it was more appropriate to use tissues from an HIV infected individual who had died of a drug overdose before the progression to AIDS. TTV and/or TLMV were found in all six of the tissues tested with bone marrow, cerebellum and liver having lower titres than spleen, thoracic spinal cord and lymph node. Interestingly, both the thoracic spinal cord and the cerebellum were infected with Anelloviruses: the spinal cord with TTV and the cerebellum with TLMV. Although it cannot be said with certainty that the virus detected was not the result of contamination with blood during collection, the presence of two different viruses within the confines of the central nervous system (CNS) makes this unlikely. The presence of TTV DNA has been demonstrated in the cerebrospinal fluid (CSF) [Maggi et al., 2001a] of both an AIDS patient and two patients without HIV, however a link with any disorder of the CNS has yet to be demonstrated.

In conclusion, this study has demonstrated that both TTV and TLMV may infect bone marrow and spleen and the titres of these viruses increases in response to the immunosuppression and decreased CD4+ T cell count associated with the progression to AIDS, implying the replication of these viruses is regulated by the immune system. The mechanisms that result in this increased viraemia warrant further investigation however it seems unlikely that the depletion of lymphocytes alone can account for this phenomenon.

Chapter 5

Titres of TTV and TLMV and Anti-TTV IgG in Sera from Immunosuppressed and Immunocompetent Individuals

5 TITRES OF TTV/TLMV AND ANTI-TTV IGG IN SERA FROM IMMUNOSUPPRESSED AND IMMUNOCOMPETENT INDIVIDUALS

5.1 Introduction

The role of the immune system in controlling TTV/TLMV infection is still widely debated. Although several studies have described increased titres in immunosuppressed individuals, such as those infected with HIV and AIDS and transplant recipients [Shibayama et al., 2001; Touinssi et al., 2001], serial samples taken from HIV positive individuals before and after progression to AIDS failed to find any increased titre on an individual level [Moen et al., 2002b].

Anti-TTV IgG and IgM have been isolated both from humans and from chimpanzees infected with human TTV recovered from the faeces of an infected individual [Kakkola et al., 2002; Tawara et al., 2000; Tsuda et al., 2001]. These limited studies investigating the antibody status of people infected with TTV/TLMV have given differing accounts regarding the sequential appearance of circulating IgG and IgM.

This chapter aims to build on the results outlined in chapter four, which investigated variation in the titre of Anelloviruses in HIV positive and negative tissue samples and showed there are significant differences between those two groups of patients. The increased titre of TTV/TLMV in response to a decreased CD4+ T cell count suggested the immune system plays a role in controlling the replication of the virus leading to a higher viral load in those whose immune system is compromised. Unlike HIV infection, infection with HCV and HBV does not lead to immunodeficiency so samples containing there markers were used in this study to represent virally infected individuals without compromised immune systems.

5.2 Results

5.2.1 Comparison of TTV/TLMV viral titres in sera from HIV, HCV or HBV positive individuals with non co-infected controls

To investigate the titre of TTV/TLMV circulating in the serum of individuals who are immunocompromised and those who are immunocompetent, ten serum samples from patients infected with either HIV, HCV or HBV, and ten serum samples from patients with none of the aforementioned viral markers were provided by Ms. Carol Lycett, Clinical

Scientist B, NAT Reference Laboratory, Scottish National Blood Transfusion Service, Royal 'Dick' Vet School, Summerhall, Edinburgh, EH9 1QH. These were anonymised and no sample data were available.

DNA was extracted from the serum samples using the Roche High Pure Viral Template kit and the viral load quantified by limiting dilution PCR, as shown in Figure 5.2.1 to Figure 5.2.4.

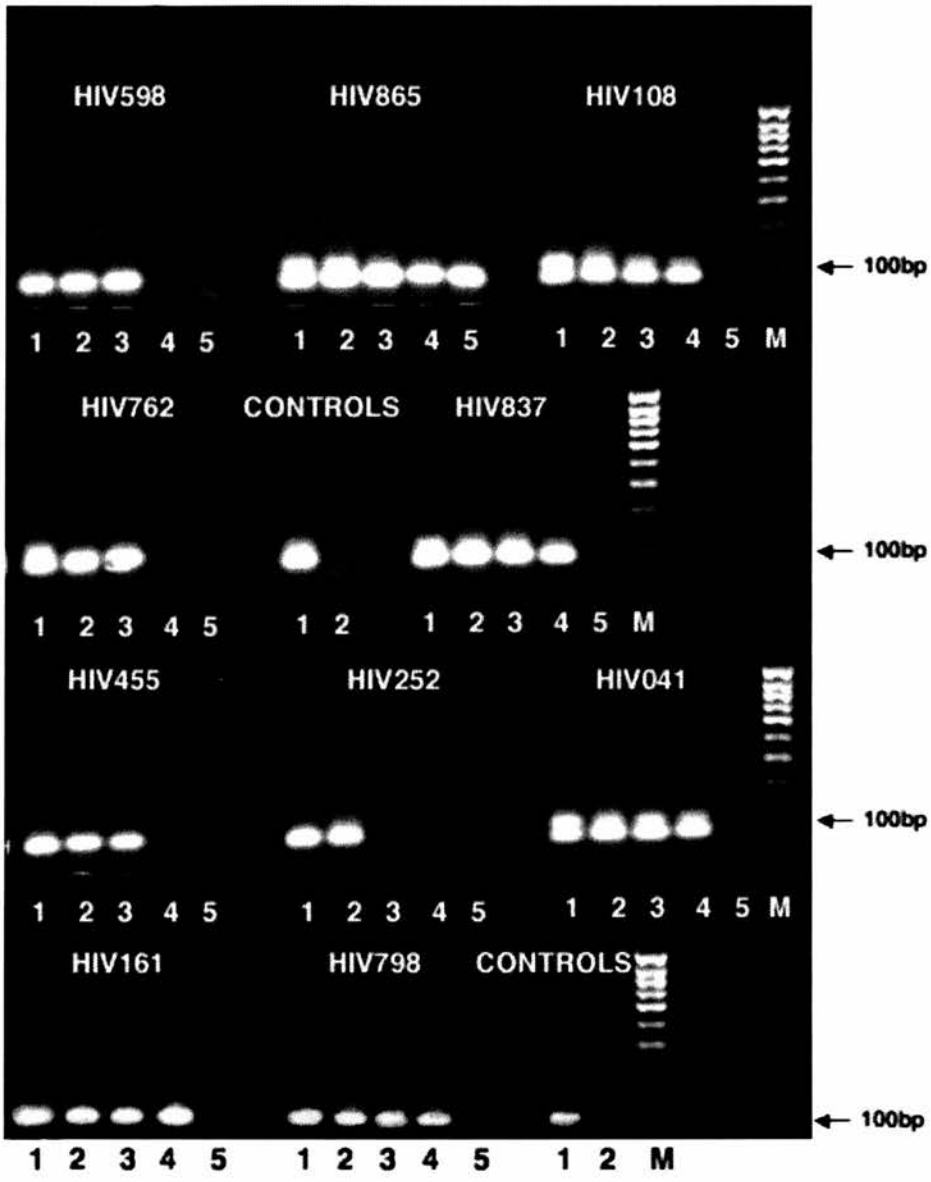

Figure 5.2.1 – UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from HIV positive serum. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: amount of DNA equivalent to 4 μl serum then 10 fold dilutions to Lane 5: amount of DNA equivalent to $4x10^{-4}$ μl serum); Lane M: 100bp DNA ladder. Controls, Lane 1: Positive control (minipool); Lane 2: Negative control (water).

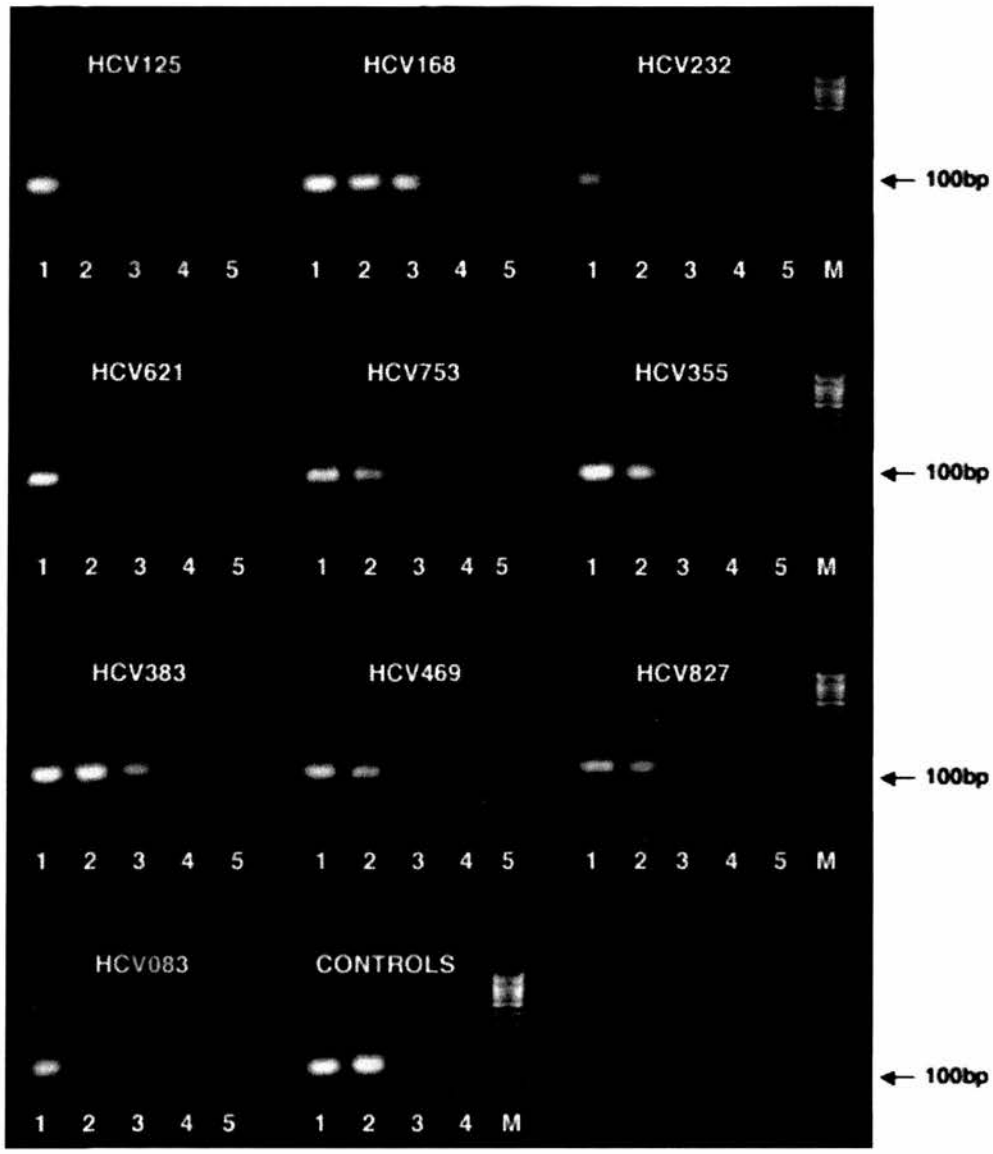

Figure 5.2.2 – UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from HCV positive serum. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: amount of DNA equivalent to 4 μl serum then 10 fold dilutions to Lane 5: amount of DNA equivalent to $4x10^5$ μl serum); Lane M: 100bp DNA ladder. Controls, Lanes 1&2: Positive controls (minipools); Lanes 3&4: Negative controls (water).

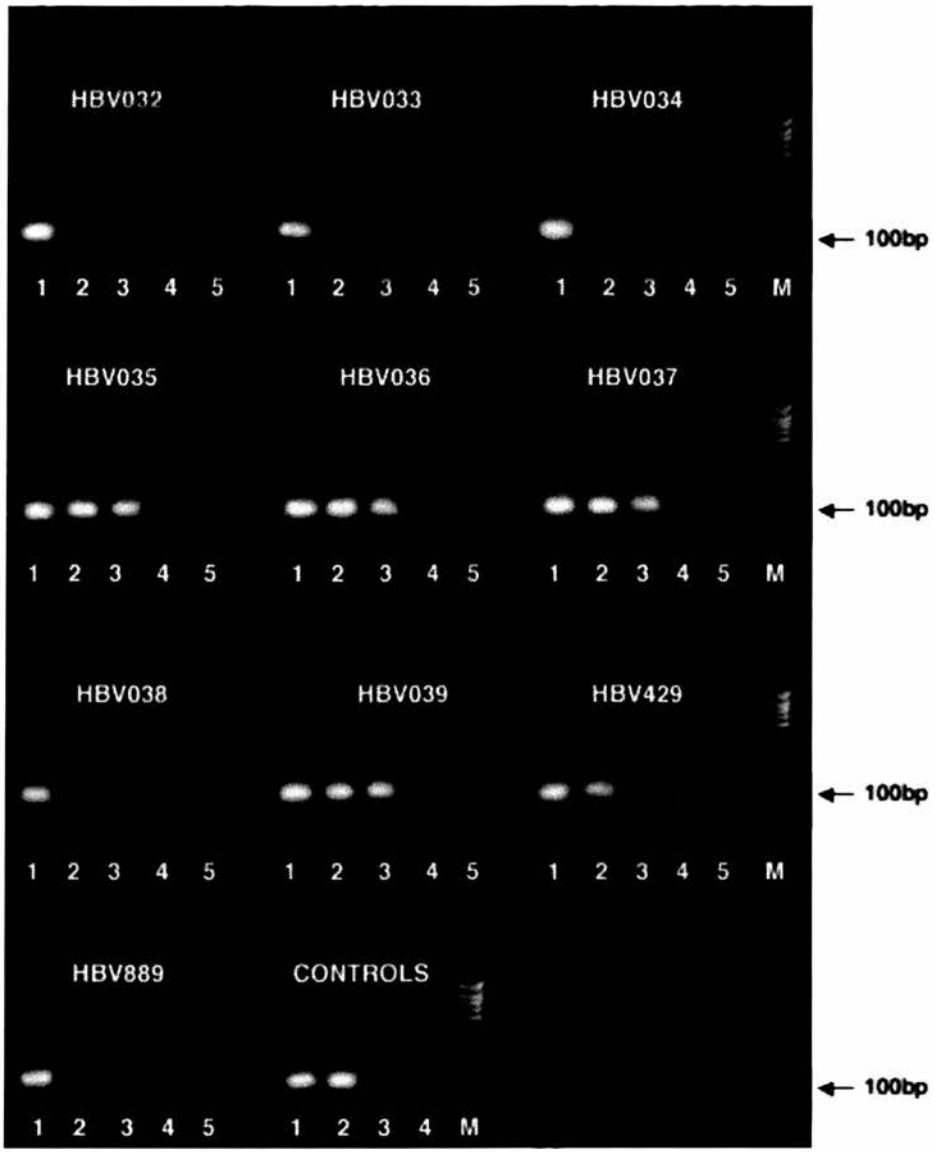

Figure 5.2.3 – UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from HBV positive serum. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: amount of DNA equivalent to 4 μl serum then 10 fold dilutions to Lane 5: amount of DNA equivalent to $4x10^{-4}$ μl serum); Lane M: 100bp DNA ladder. Controls, Lanes 1&2: Positive controls (minipools); Lanes 3&4: Negative controls (water).

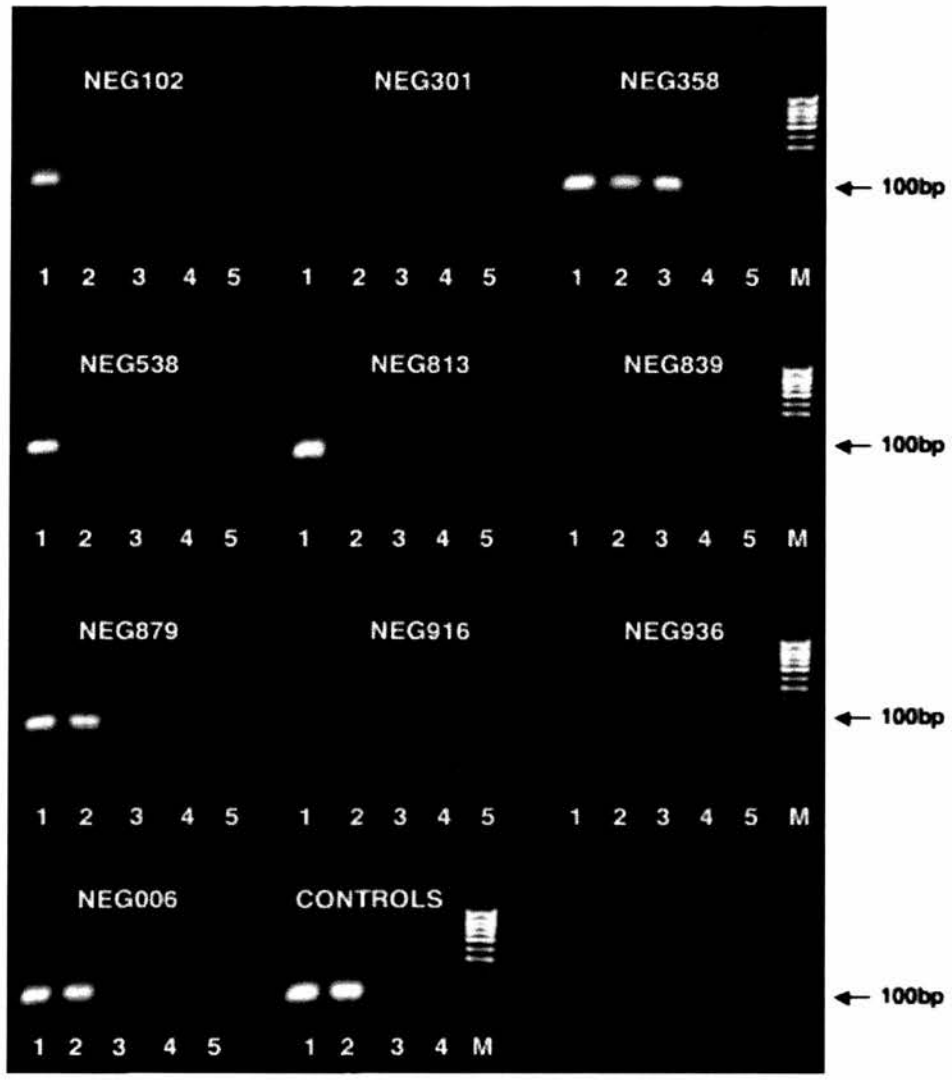

Figure 5.2.4 – UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from HIV, HCV and HBV negative serum. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: amount of DNA equivalent to 4 μl serum then 10 fold dilutions to Lane 5: amount of DNA equivalent to $4x10^{-4}$ μl serum); Lane M: 100bp DNA ladder. Controls, Lanes 1&2: Positive controls (minipools); Lanes 3&4: Negative controls (water).

The last positive dilution was used to estimate the number of copies of TTV/TLMV per ml of serum, as shown in Table 5.2.1. All of the serum samples taken from individuals who were infected with either HIV, HCV or HBV were PCR positive for TTV/TLMV although co-infection with HIV was associated with the highest titre of Anelloviruses, as shown in Figure 5.2.5 (HIV positive sera: mean 382750 copies per ml, median 250000 copies per ml; HCV positive sera: mean 6100 copies per ml, median 2500 copies per ml; HBV positive sera: mean 10375 copies per ml, median 1375 copies per ml).

HIV positive serum sample	Number	Number of copies of TTV/TLMV per ml of serum	HCV positive serum sample	Number of copies of TTV/TLMV per ml of serum	HBV positive serum samples	Number of copies of TTV/TLMV per ml of serum	HIV/HCV/HBV negative serum sample	Number of copies of TTV/TLMV per ml of serum
1	HIV598	25000	HCV125	250	HBV032	250	NEG102	250
2	HIV865	2500000	HCV168	25000	HBV033	250	NEG301	0
3	HIV108	250000	HCV232	250	HBV034	250	NEG358	25000
4	HIV762	25000	HCV621	250	HBV035	25000	NEG538	250
5	HIV837	250000	HCV753	2500	HBV036	25000	NEG813	250
6	HIV455	25000	HCV355	2500	HBV037	25000	NEG839	0
7	HIV252	2500	HCV383	25000	HBV038	250	NEG879	2500
8	HIV041	250000	HCV469	2500	HBV039	25000	NEG916	0
9	HIV161	250000	HCV827	2500	HBV429	2500	NEG936	0
10	HIV798	250000	HCV083	250	HBV889	250	NEG006	2500

Table 5.2.1 - Estimation of the number of copies of TTV/TLMV per ml of serum from HIV, HCV, HBV positive individuals and HIV/HCV/HBV negative individuals. 'Number' corresponds to numbers on the X-axis of the graph shown in Figure 5.2.5.

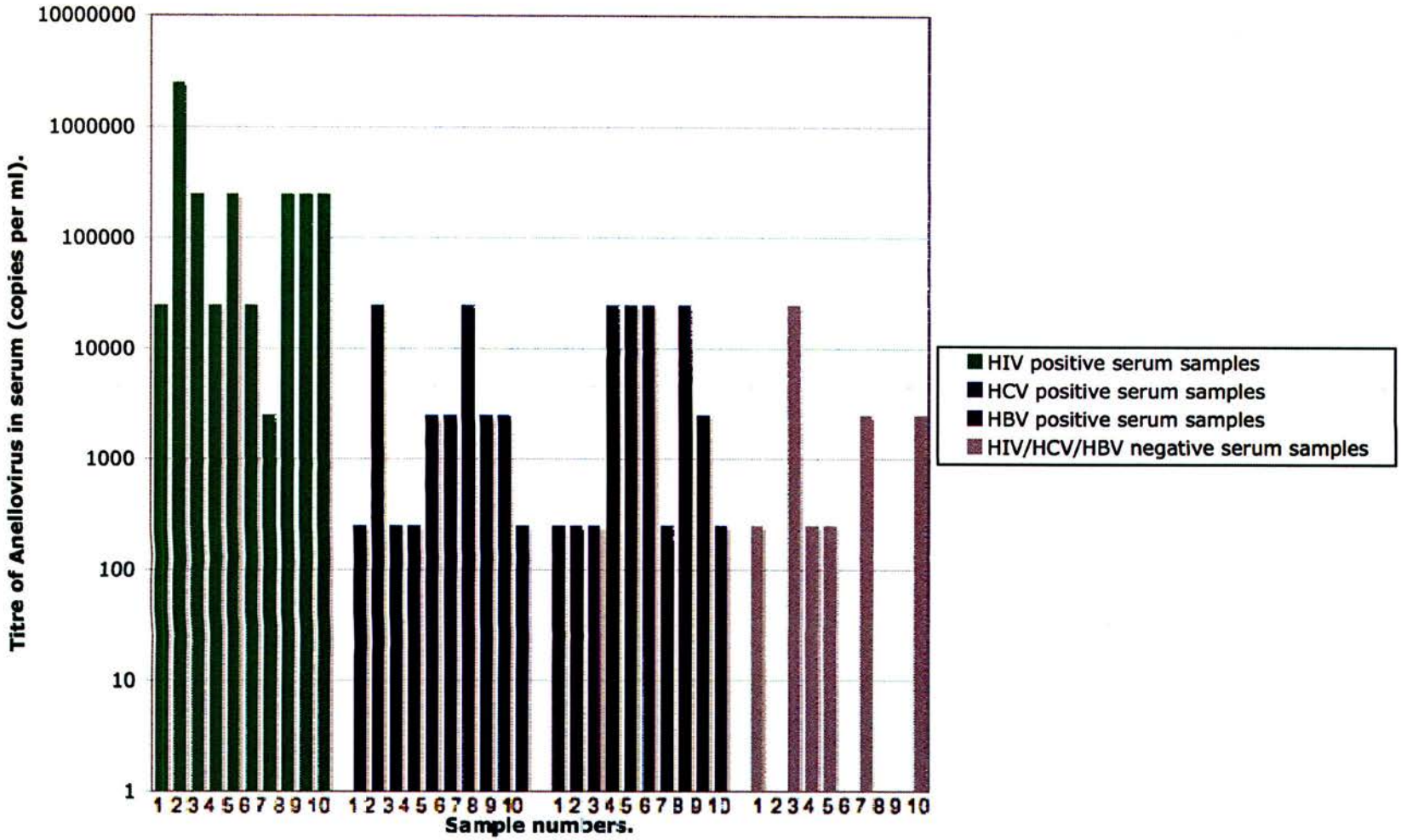

Figure 5.2.5 – Comparison of titre of Anelloviruses in the serum of HIV, HCV and HBV positive individuals and HIV, HCV and HBV negative individuals.

Of the ten sera collected from patients with no known viral infection, four were PCR negative for TTV/TLMV. The six that were PCR positive had a mean of 5125 copies per ml and a median of 1375 copies per ml.

The Kruskal-Wallis test was used to test for differences between the four groups. A p-value of <0.001 was obtained, indicating significant differences, however the test is unable to indicate between which groups the significant differences occur. In order to ascertain this, each pair of samples was subjected to analysis using the Mann-Whitney U-test. The p-values for each pair of samples is shown in Table 5.2.2.

Comparison of the titre of TTV/TLMV in sera using the Mann-Whitney U-test	p-value
HIV/HCV/HBV negative sera vs. HBV positive sera	0.063
HIV/HCV/HBV negative sera vs. HCV positive sera	0.07
HIV/HCV/HBV negative sera vs. HIV positive sera	<0.001
HBV positive sera vs. HCV positive sera	0.871
HBV positive sera vs. HIV positive sera	0.002
HCV positive sera vs. HIV positive sera	0.001

Table 5.2.2 – p-values from Mann-Whitney U-test used to compare titres of TTV/TLMV in serum. The null hypothesis is there is no significant difference of viral titre between the two groups. Significant p-values where the null hypothesis can be rejected are shown in red.

The table shows a notably greater titre of TTV/TLMV in the sera of HIV positive individuals compared with the groups infected with other viruses and those with no known viral infection. Although the difference in titre between the control group (HIV/HCV/HBV negative) and the HCV and HBV positive groups is not statistically significant, the p-values of 0.063 (HBV) and 0.07 (HCV) suggest there is trend towards Anellovirus titre that is higher when infection with TTV/TLMV is combined with a second virus.

5.2.2 TTV/TLMV titres in sera from transplant recipients

To compare the titre of TTV/TLMV in sera taken from HIV positive patients with a second group of patients who were immunocompromised, the TTV/TLMV titre in the serum of 20 anonymised patients who were receiving of immunosuppressive drug treatment were analysed. Serum samples from patients who had recently undergone either a heart transplant, a lung transplant or a heart and lung transplant (mean 47.9 days post transplant, median 45.5 days post transplant), were provided by Dr Paul Hopwood, Department on Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh.

DNA was extracted from the sera using the Roche High Pure Viral Template kit and the titre of TTV/TLMV estimated using limiting dilution PCR (Figure 5.2.6).

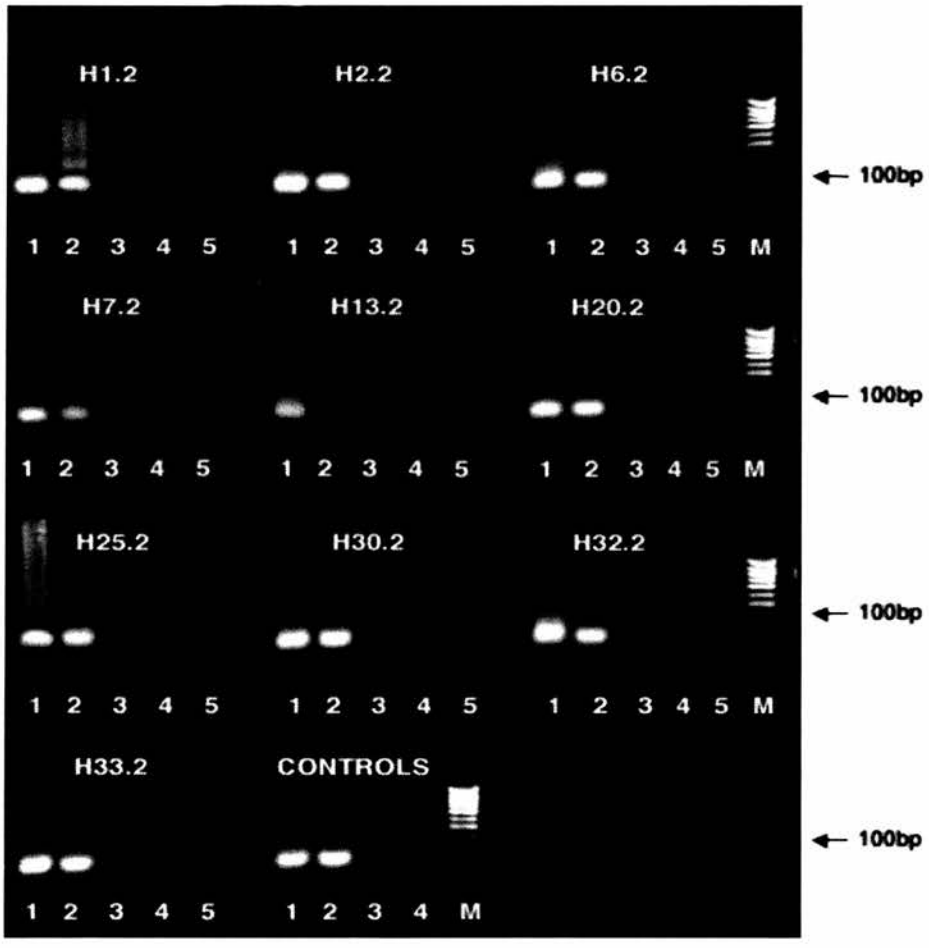

Figure 5.2.6 - UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from serum taken from transplant recipients. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: amount of DNA equivalent to 4 μl serum then 10 fold dilutions to Lane 5: amount of DNA equivalent to $4x10^{-4}$ μl serum); Lane M: 100bp DNA ladder. Controls, Lanes 1&2: Positive controls (minipools); Lanes 3&4: Negative controls (water).

The last PCR positive dilution was used to estimate the number of virus copies present in one ml of serum, as shown in Table 5.2.3 (mean: 3512.5 copies per ml; median: 2500 copies per ml). The table shows a remarkable consistency in the titre of virus in the serum of these individuals. With the exception of samples H13.2 which contained 250 copies per ml and H36.2, containing 25000 copies per ml, all of the serum samples contained 2500 copies of TTV/TLMV per ml.

Following the transplant, patients were subjected to immunosuppressive drug therapy to artificially suppress the immune system and prevent rejection of the transplanted organ. Combinations of the corticosteroids prednisolone and methylprednisolone, the cytotoxic drug azathioprine, and cylosporin A, a calcineurin inhibitor, were used to prevent rejection. A graph indicating the doses of these drugs in relation to the TTV/TLMV titre is shown in Figure 5.2.7.

To investigate if any of the drugs influenced the titre of TTV/TLMV in the serum of the patients, linear regression was carried out. No significant association was found between the viral load and the drug dose for azathioprine, prednisolone and methylprednisolone ($p>0.2$). There was also no significant association with the cumulative drug dose taken up to the point the serum sample was collected ($p>0.5$). Although the association between Anellovirus titre and cyclosporin A dose was not significant (p-value=0.088), there does appear to be a trend towards this.

Number	Transplant sample	Days post transplant (days)	Organ(s) transplanted	Number of copies of TTV/TLMV per ml of serum
1	H1.2	36	Heart	2500
2	H2.2	35	Heart	2500
3	H6.2	34	Lung	2500
4	H7.2	29	Lung	2500
5	H13.2	28	Lung	250
6	H20.2	45	Lung	2500
7	H25.2	61	Lung	2500
8	H30.2	56	Lung	2500
9	H32.2	48	Heart	2500
10	H33.2	39	Lung	2500
11	H36.2	56	Heart	25000
12	H39.2	46	Heart and lung	2500
13	H41.2	37	Lung	2500
14	H46.2	107	Heart	2500
15	H51.2	57	Heart and lung	2500
16	H60.2	70	Lung	2500
17	H61.1	21	Heart	2500
18	H87.2	57	Lung	2500
19	H88.2	56	Heart and lung	2500
20	H92.2	41	Heart and lung	2500

Table 5.2.3 - Estimation of the number of copies of TTV/TLMV per millilitre of serum from transplant patients. 'Number' corresponds to the number on the x-axis of Figure 5.2.7.

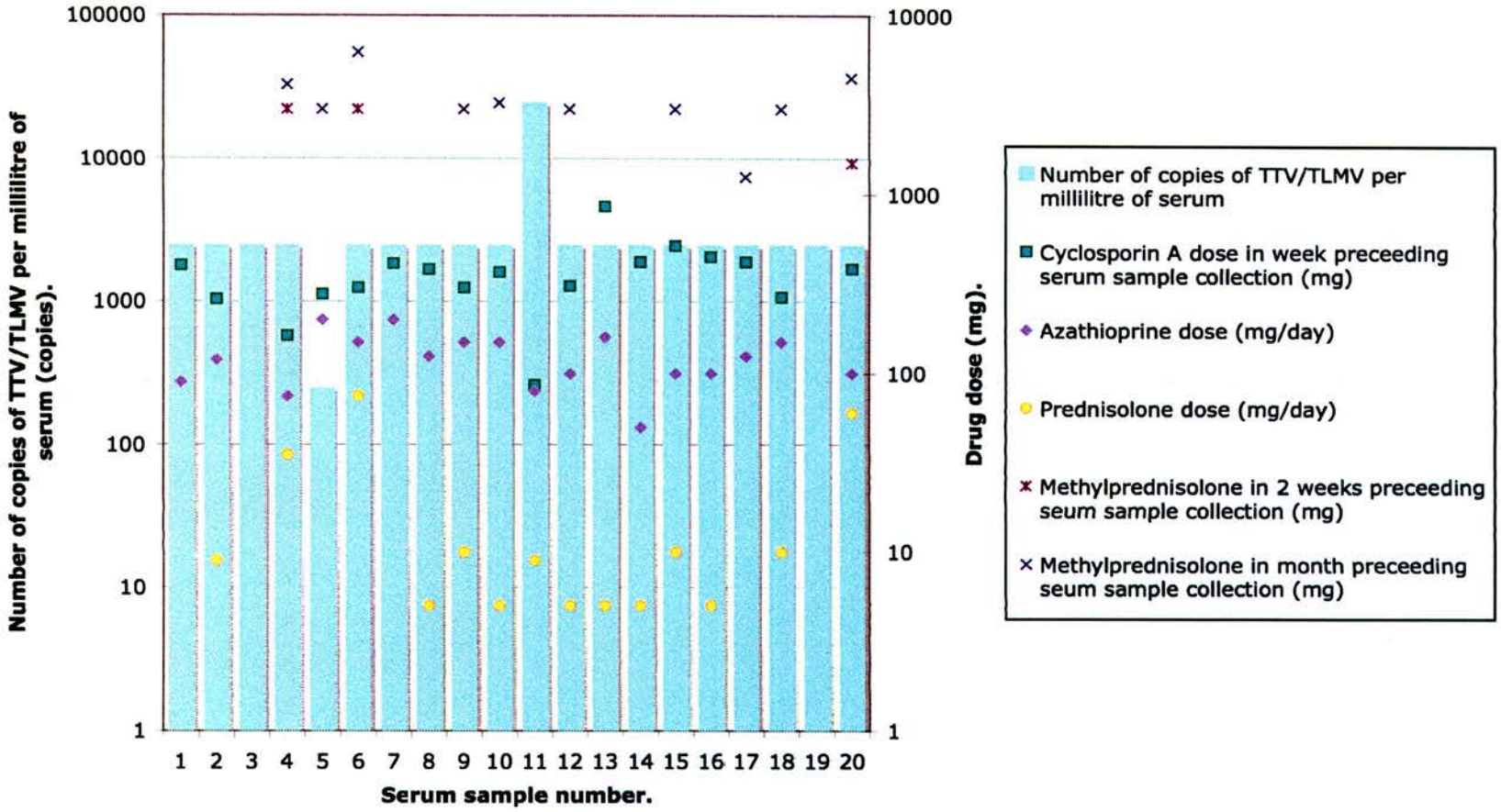

Figure 5.2.7 – Number of copies of TTV/TLMV per ml of serum from transplant patients shown with details of immunosuppressive drug combination for each sample.

5.2.2.1 Titre of TTV/TLMV in the serum of transplant patients compared with TTV/TLMV titre in HIV, HCV or HBV positive or HIV/HCV/HBV negative sera

The Mann-Whitney U-test was used to compare the titre of TTV/TLMV in the serum of transplant patients with the titre of virus in serum from HIV, HCV and HBV positive individuals and HIV, HCV and HBV negative serum (Section 5.2.1). The p-values are shown in Table 5.2.4. As the table shows, the HCV, HBV and HIV negative serum samples have significantly less TTV/TLMV and the HIV positive serum samples have a significantly higher titre of TTV/TLMV than the transplant sera.

Comparison of the titre of TTV/TLMV in sera using the Mann-Whitney U-test	p-value
Sera from transplant patients vs. HIV/HCV/HBV negative sera	0.002
Sera from transplant patients vs. HCV positive sera	0.281
Sera from transplant patients vs. HBV positive sera	0.626
Sera from transplant patients vs. HIV positive sera	<0.001

Table 5.2.4 - p-values from Mann-Whitney U-test used to compare titres of TTV/TLMV in serum. The null hypothesis is there is no significant difference of viral titre between the two groups. Significant p-values where the null hypothesis can be rejected are shown in red.

5.2.3 Difference in proportion of TTV/TLMV bound to IgG and free TTV/TLMV in sera infected with HIV, HCV, HBV, non-infected controls and transplant patients

HIV positive sera appears to contain a much higher titre of TTV/TLMV than sera infected with other viral agents (HBV and HCV) and sera taken from transplants patients who are subjected to immunosuppressive drug regimes. Although yet to be proved conclusively, it is possible that acquired immunodeficiency associated with HIV infection and the progression towards AIDS in some way reduces the immune response directed towards TTV/TLMV and TTV/TLMV infected cells and results in unchecked replication of these

viruses. To investigate the possibility of a decreased antibody response in immunocompromised patients, the proportion of virus bound to IgG was compared with the proportion of free virus in the sera of five groups of individuals (HIV positive, HCV positive, HBV positive, HIV, HCV and HBV negative and transplant recipients) as an indicator of the antibody response to the Anelloviruses.

IgG present in the ten sera samples from each of the five groups was captured using protein G magnetic beads. Nucleic acid was extracted from IgG depleted serum and the protein G bead eluate using the QIAamp Minelute Virus Spin kit and the TTV/TLMV titre semi-quantitated using limiting dilution PCR. Representative agarose gels are shown in Figure 5.2.8 (IgG depleted serum) and Figure 5.2.9 (IgG).

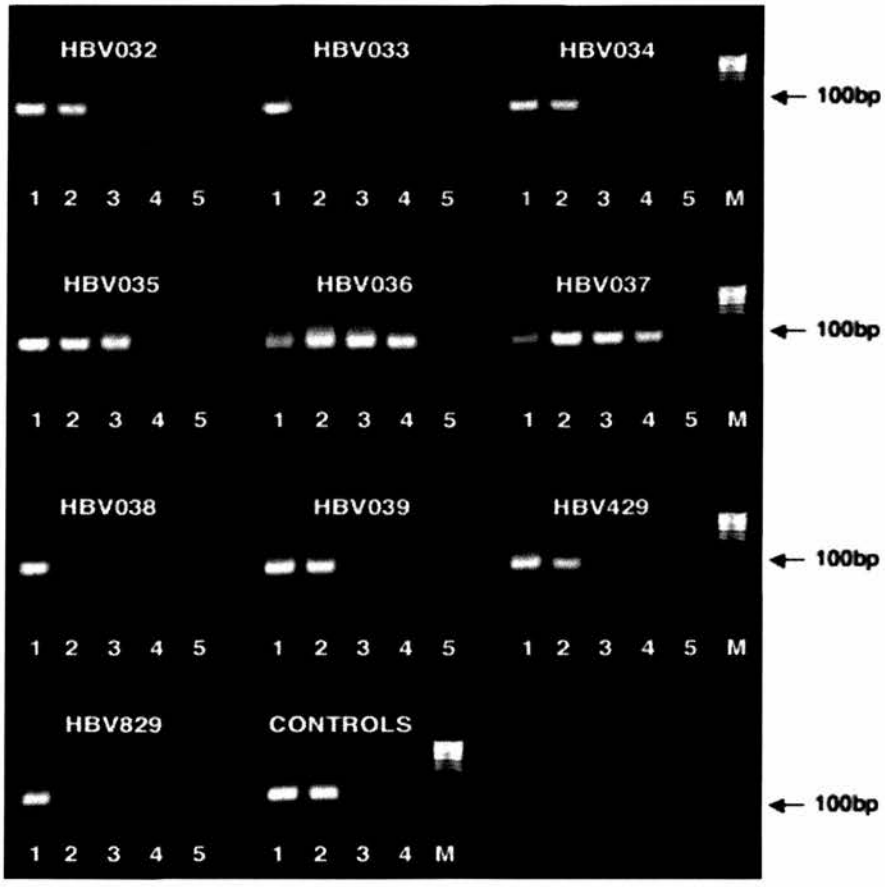

Figure 5.2.8 - UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from IgG depleted HBV positive serum. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: 2 µl DNA then 10 fold dilutions to Lane 5: $2x10^{-4}$ µl DNA); Lane M: 100bp DNA ladder. Controls, Lanes 1&2: Positive controls (minipools); Lanes 3&4: Negative controls (water).

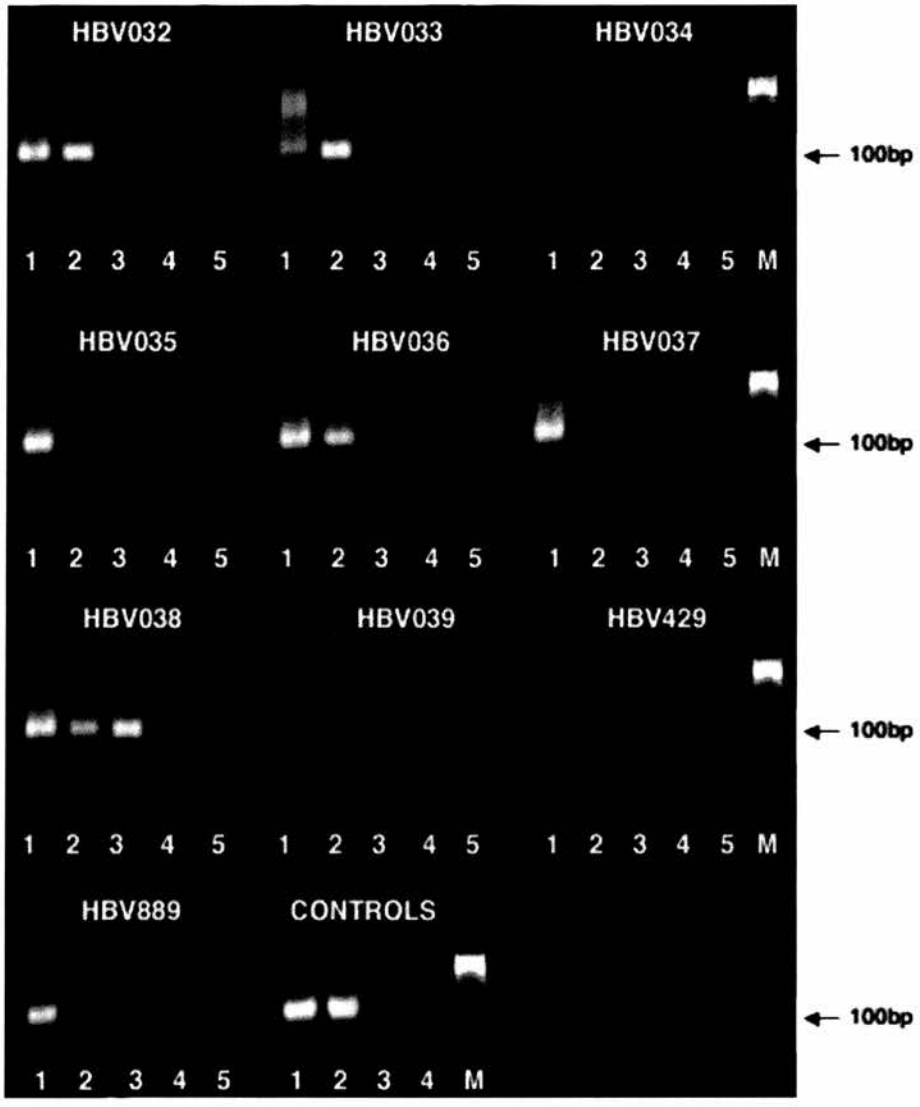

Figure 5.2.9 - UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from IgG isolated from HBV positive serum. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: 2 μl DNA then 10 fold dilutions to Lane 5: $2x10^{-4}$ μl DNA); Lane M: 100bp DNA ladder. Controls, Lanes 1&2: Positive controls (minipools); Lanes 3&4: Negative controls (water).

The number of PCR positive dilutions from the IgG depleted serum and the isolated IgG were compared for each sample as shown in Figure 5.2.10 to Figure 5.2.14. These graphs show a wide range of variation in the proportion of virus bound to IgG compared with free virus in all five of the groups. Interestingly, the HIV, HCV and HBV negative group is the only one of the five groups that included samples which contained TTV/TLMV bound to IgG with no evidence of free virus.

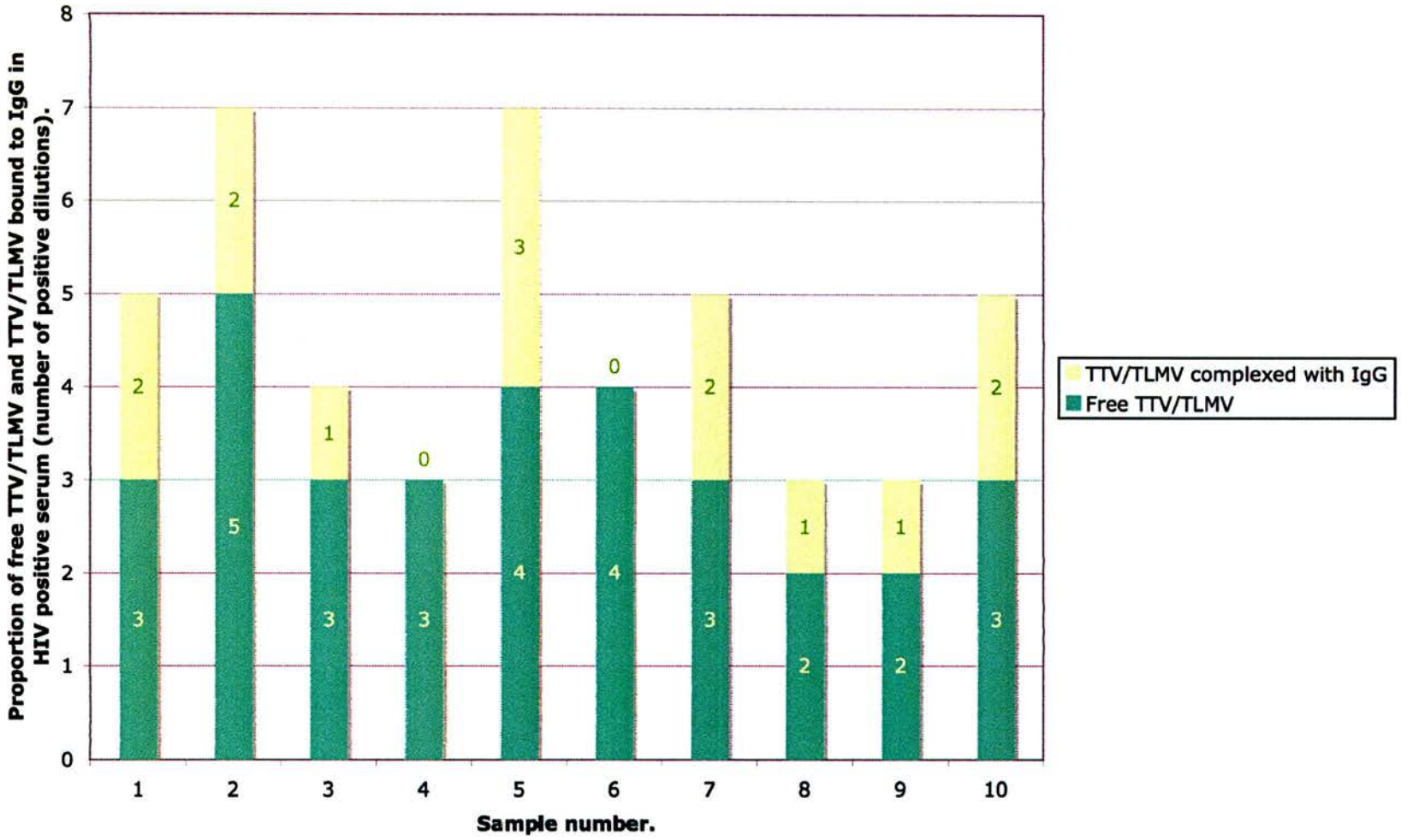

Figure 5.2.10 – Graph depicting the proportion of TTV/TLMV bound to IgG compared with the proportion of free virus in the serum of HIV positive individuals. The number of positive dilutions of complexed and free virus as indicated by limiting dilution PCR using UTR primers are shown as digits on the graph.

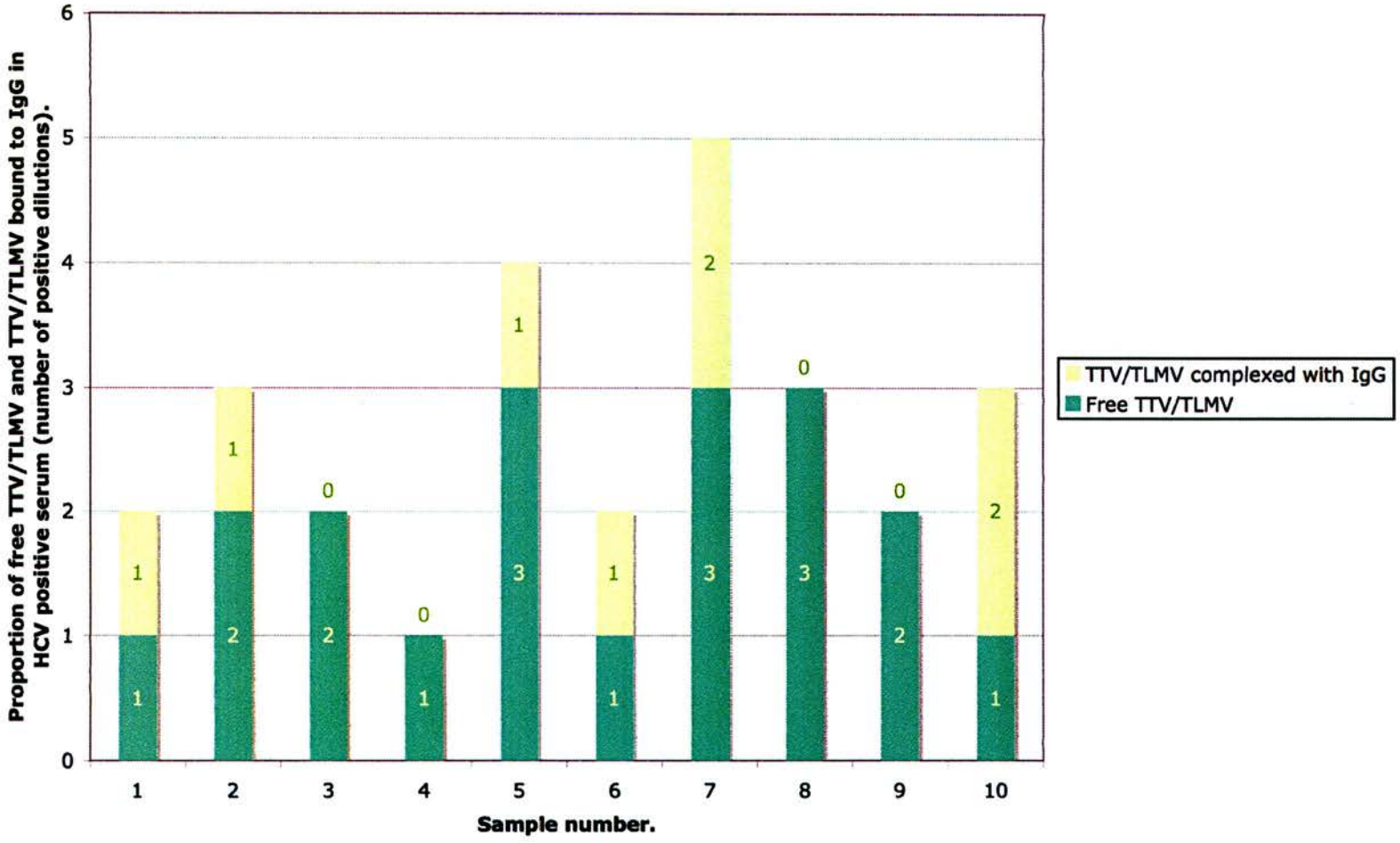

Figure 5.2.11 - Graph depicting the proportion of TTV/TLMV bound to IgG compared with the proportion of free virus in the serum of HCV positive individuals. The number of positive dilutions of complexed and free virus as indicated by limiting dilution PCR using UTR primers are shown as digits on the graph.

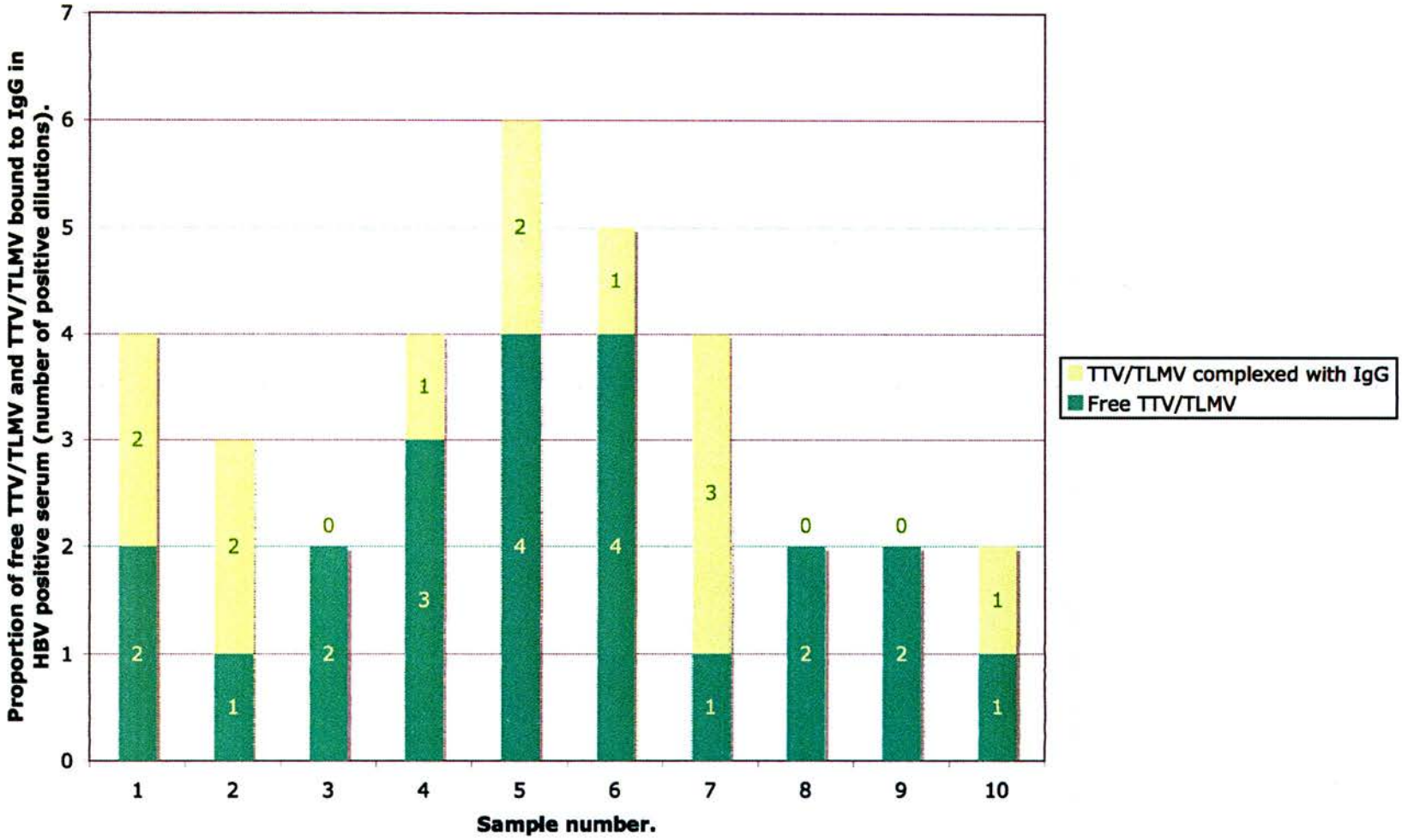

Figure 5.2.12 - Graph depicting the proportion of TTV/TLMV bound to IgG compared with the proportion of free virus in the serum of HBV positive individuals. The number of positive dilutions of complexed and free virus as indicated by limiting dilution PCR using UTR primers are shown as digits on the graph.

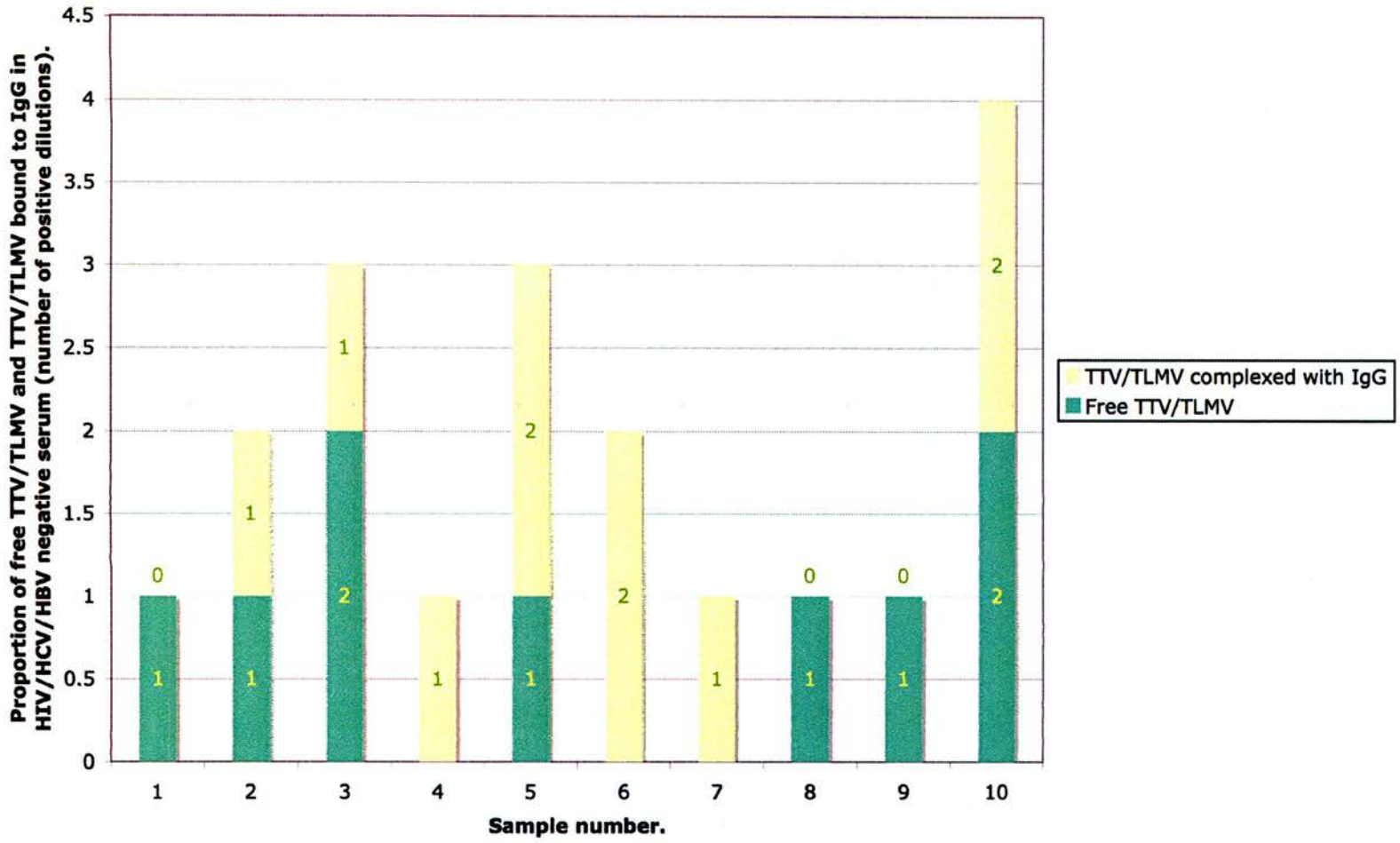

Figure 5.2.13 - Graph depicting the proportion of TTV/TLMV bound to IgG compared with the proportion of free virus in HIV, HCV and HBV negative serum. The number of positive dilutions of complexed and free virus as indicated by limiting dilution PCR using UTR primers are shown as digits on the graph. Sample numbers 4, 6 and 7 had no detectable free virus.

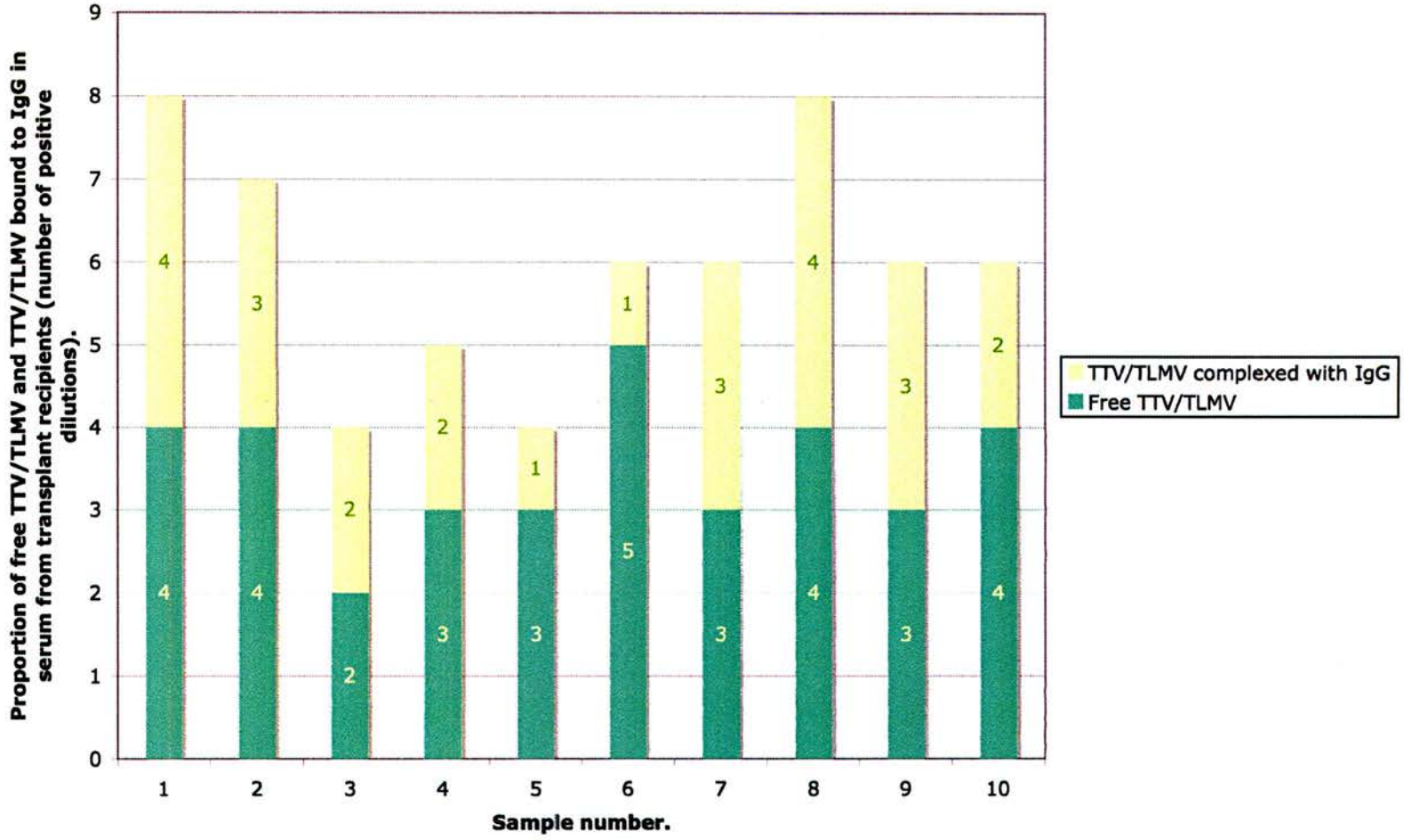

Figure 5.2.14 - Graph depicting the proportion of TTV/TLMV bound to IgG compared with the proportion of free virus in serum from transplant recipients. The number of positive dilutions of complexed and free virus as indicated by limiting dilution PCR using UTR primers are shown as digits on the graph.

The proportions of bound and unbound virus were calculated by taking the difference in the number of PCR positive dilutions between the free virus found in the IgG depleted serum fraction and the virus complexed with IgG from the isolated IgG fraction (free virus – complexed virus, as shown in Table 5.2.5). The mean value for each group, shown in the bottom row of the table, indicates that all of the groups have a higher proportion of free virus then virus complexed with IgG. The exception to this is the HIV, HCV and HBV negative group which has a mean value of -0.1, showing that in these individuals there is a higher proportion of virus bound to IgG than free virus.

To investigate if proportions of free TTV/TLMV versus TTV/TLMV bound to IgG were significantly different between the five groups, the Kruskal-Wallis test, which assumes there is no significant difference between the groups, was carried out. The p-value for this test was 0.048, indicating the null hypothesis could be rejected in favour of the alternative hypothesis that there is significant differences. The Mann-Whitney test was then used on pairs of groups using the same null hypothesis, as shown in Table 5.2.6. The test shows there are significantly different proportions of bound and unbound TTV/TLMV between the HIV, HCV and HBV negative group and the HIV positive and HCV positive groups (p-values < 0.05).

Sample	HIV positive serum (free TTV/TLMV titre – titre of TTV/TLMV bound to IgG)	HCV positive serum (free TTV/TLMV titre – titre of TTV/TLMV bound to IgG)	HBV positive serum (free TTV/TLMV titre – titre of TTV/TLMV bound to IgG)	HIV, HCV and HBV negative serum (free TTV/TLMV titre – titre of TTV/TLMV bound to IgG)	Transplant recipient serum (free TTV/TLMV titre – titre of TTV/TLMV bound to IgG)
1	1	0	0	1	0
2	3	1	-1	0	1
3	2	2	2	1	0
4	3	1	2	-1	1
5	1	2	2	-1	2
6	4	0	3	-2	4
7	1	1	-2	-1	0
8	1	3	2	1	0
9	1	2	2	1	0
10	1	-1	0	0	2
Mean	1.8	1.1	1	-0.1	1

Table 5.2.5 – Difference between the number of PCR positive dilutions of unbound TTV/TLMV and TTV/TLMV bound to IgG (free TTV/TLMV – titre of TTV/TLMV bound to IgG) as determined by capture of IgG from serum using magnetic beads followed by limiting dilution TTV/TLMV PCR using nucleic acid extracted from the IgG depleted serum and captured IgG.

Comparison of free TTV/TLMV – TTV/TLMV complexed with IgG in serum using the Mann-Whitney U-test	p-value
HIV positive sera vs. HCV positive sera	0.282
HIV positive sera vs. HBV positive sera	0.460
HIV positive sera vs. HIV, HCV and HBV negative sera	0.002
HIV positive sera vs. sera from transplant recipients	0.090
HCV positive sera vs. HBV positive sera	0.906
HCV positive sera vs. HIV, HCV and HBV negative sera	0.039
HCV positive sera vs. sera from transplant recipients	0.638
HBV positive sera vs. HIV, HCV and HBV negative sera	0.076
HBV positive sera vs. sera from transplant recipients	0.752
HIV, HCV and HBV negative sera vs. sera from transplant recipients	0.099

Table 5.2.6 - p-values from Mann-Whitney U-test used to compare free TTV/TLMV with TTV/TLMV bound to IgG in serum. The null hypothesis is there is no significant difference in proportions between the two groups. Significant p-values where the null hypothesis can be rejected are shown in red.

5.2.4 Titre of TTV/TLMV in sequential serum samples from transplant recipients

The titre of TTV/TLMV in the serum of 20 patients who had recently received a transplant was investigated in section 5.2.2, however these samples appeared to lack the variability of titre seen in the other groups studied, with 90% of transplant recipients having 2500 copies of virus per ml of serum. It also showed a significant difference between the titre of TTV/TLMV in transplant patients compared with both HIV positive and HIV, HCV and HBV negative individuals. To explore the dynamics of TTV/TLMV infection in individuals post transplant, sequential serum samples from two transplant recipients over an extended period of time (524 days and 917 days) were provided by Dr. Paul Hopwood and examined for changes in the virus titre.

Nucleic acid was extracted from serum samples from lung transplant recipients using the Roche High Pure Viral Template kit and the TTV/TLMV titre estimated using limiting dilution PCR (Figure 5.2.15 and Figure 5.2.16). The last positive dilution was used to estimate the number of copies of TTV/TLMV per ml of serum for both patients (Table 5.2.7 and Table 5.2.8). The tables show that the titre of virus varies over time and although the initial titre of virus in serum (at day 21 post transplant for patient H10 and day 69 post transplant for patient H21) varies by three logs between the two patients, the titre increases to peak at 25000000 copies per ml of virus at day 217 (H10) and day 251 (H21).

The change in titre of TTV/TLMV over time is shown with the doses of immunosuppressive drugs in Figure 5.2.17 and Figure 5.2.18. Although the graphs to not appear to indicate any relationship between drug dose and virus titre, linear regression was used to test for any relationship between cyclosporine A, azathioprine and prednisolone, and TTV/TLMV titre. The p-values for these test were all greater that 0.05, indicating no relationship between drug dose and virus titre.

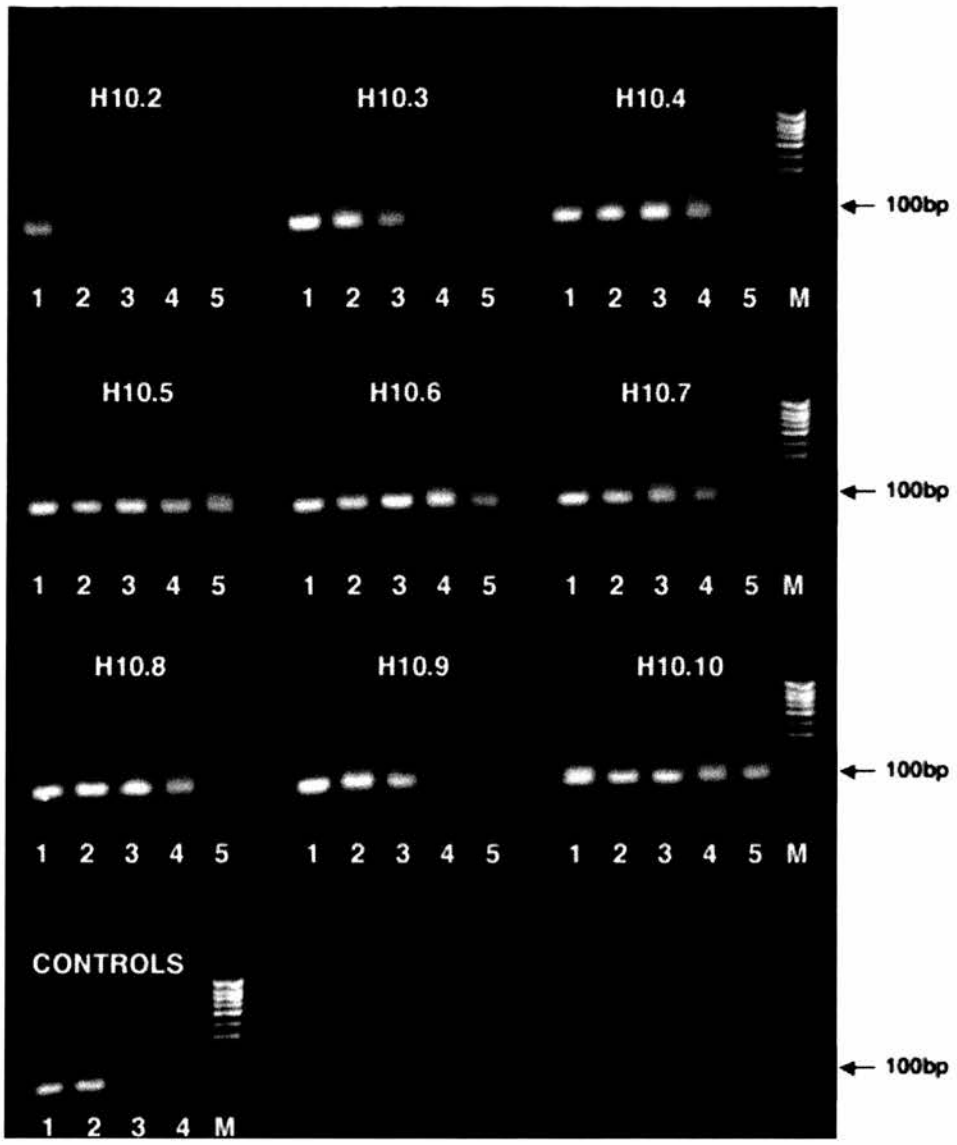

Figure 5.2.15 - UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from lung transplant recipient H10. Sample numbers are shown above each row of the agarose gel. The number after the decimal point is indicative of the sample collection time point. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: amount of DNA equivalent to 4 μl serum then 10 fold dilutions to Lane 5: amount of DNA equivalent to $4x10^{-4}$ μl serum); Lane M: 100bp DNA ladder. Controls, Lane 1 and 2: Positive controls (minipools); Lane 3 and 4: Negative controls (water).

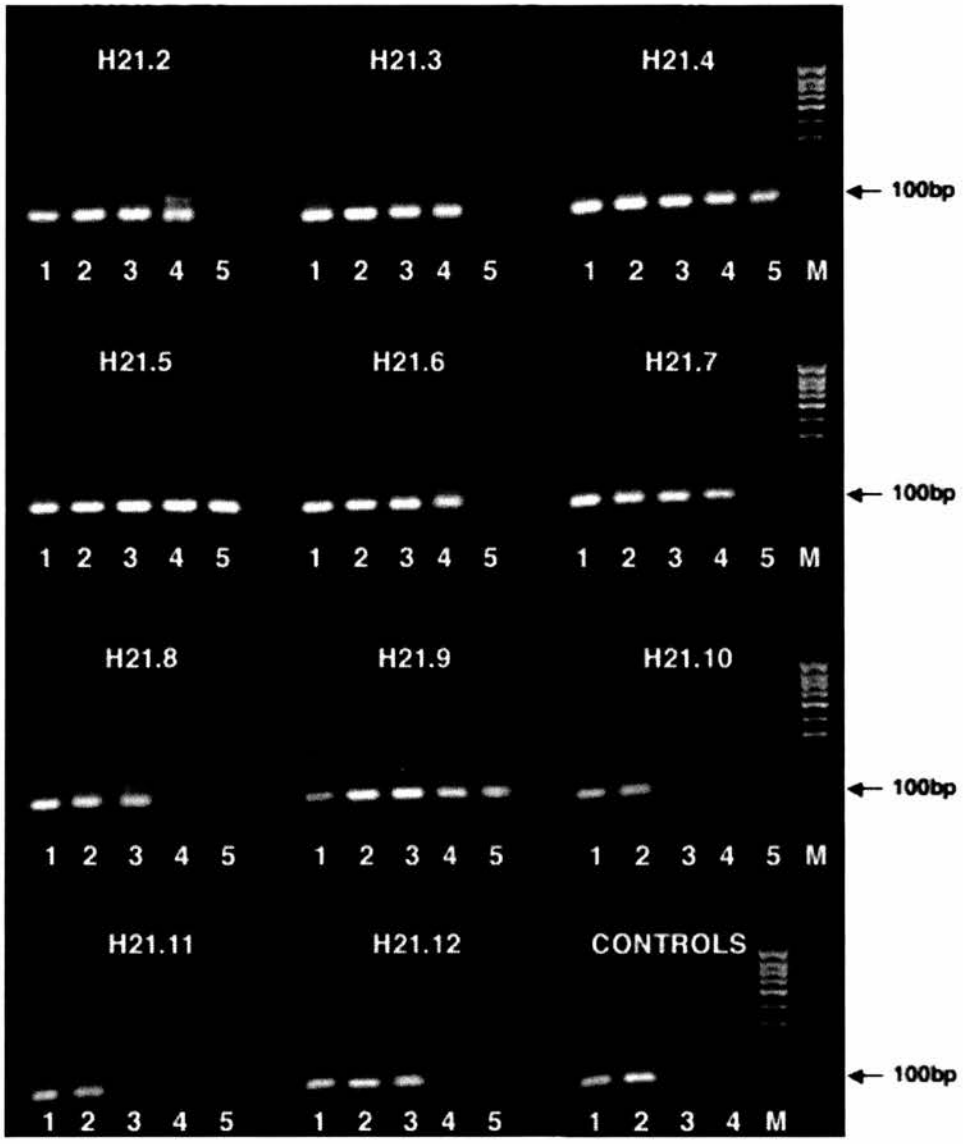

Figure 5.2.16 - UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from lung transplant recipient H21. Sample numbers are shown above each row of the agarose gel. The number after the decimal point is indicative of sample collection time point. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: amount of DNA equivalent to 4 μl serum then 10 fold dilutions to Lane 5: amount of DNA equivalent to $4x10^{-4}$ μl serum); Lane M: 100bp DNA ladder. Controls, Lane 1 and 2: Positive controls (minipools); Lane 3 and 4: Negative controls (water).

Number	Transplant sample number	Days post transplant (days)	Number of copies of TTV/TLMV per ml of serum
1	H10.2	21	250
2	H10.3	124	25000
3	H10.4	155	250000
4	H10.5	217	25000000
5	H10.6	251	2500000
6	H10.7	309	250000
7	H10.8	334	250000
8	H10.9	411	25000
9	H10.10	524	25000000

Table 5.2.7 - Estimation of the number of copies of TTV/TLMV per ml of serum from lung transplant recipient H10. 'Number' corresponds to the x-axis of Figure 5.2.17.

Number	Transplant sample number	Days post transplant (days)	Number of copies of TTV/TLMV per ml of serum
1	H21.2	69	250000
2	H21.3	125	250000
3	H21.4	181	2500000
4	H21.5	251	25000000
5	H21.6	378	250000
6	H21.7	432	250000
7	H21.8	483	25000
8	H21.9	551	2500000
9	H21.10	588	2500
10	H21.11	753	2500
11	H21.12	917	25000

Table 5.2.8 - Estimation of the number of copies of TTV/TLMV per ml of serum from lung transplant recipient H21. 'Number' corresponds to the x-axis of Figure 5.2.18.

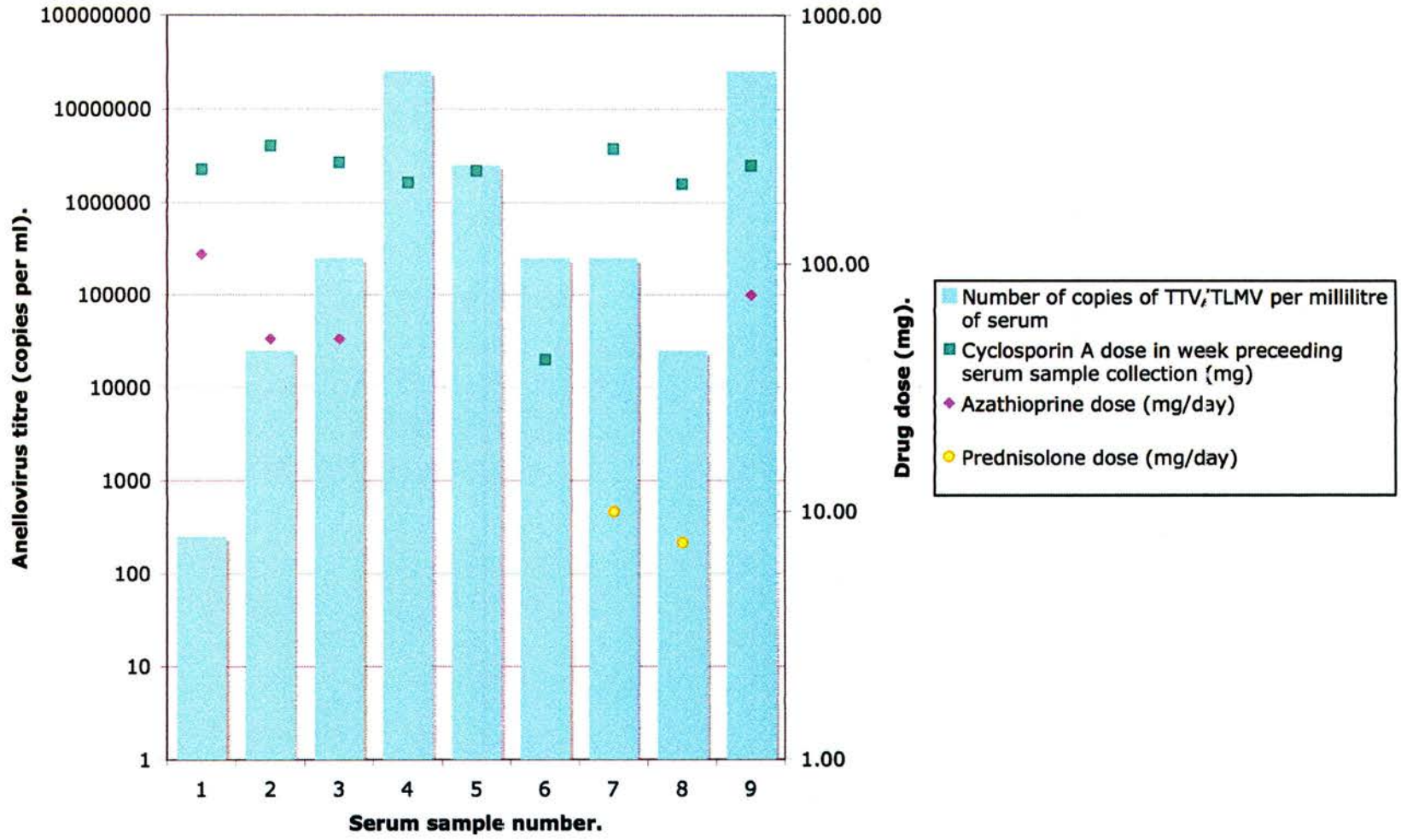

Figure 5.2.17 - Number of copies of TTV/TLMV per ml of serum taken over a period of 524 days post transplant from lung transplant patient H10 shown with details of immunosuppressive drug regime.

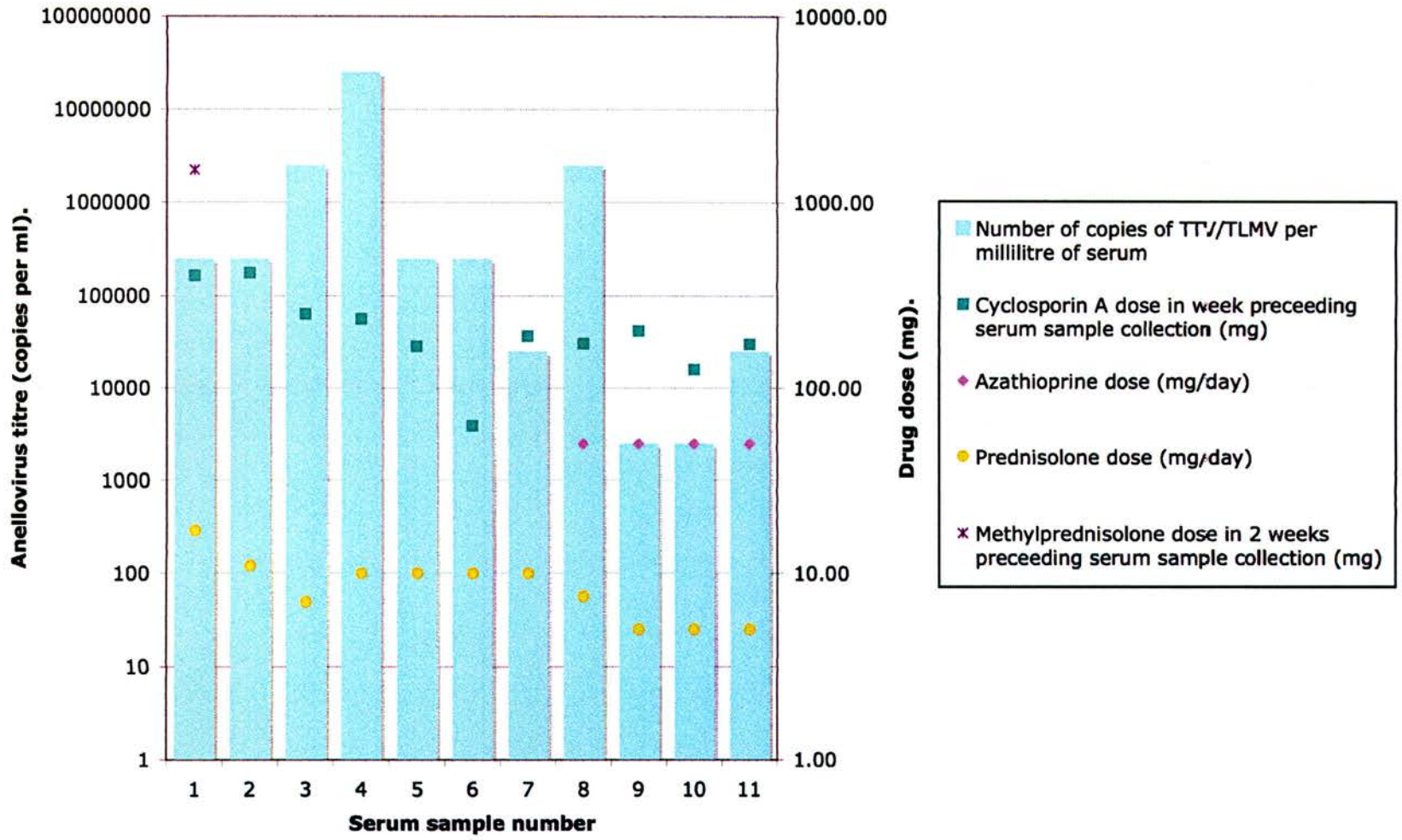

Figure 5.2.18 - Number of copies of TTV/TLMV per ml of serum taken over a period of 917days post transplant from lung transplant patient H21 shown with details of immunosuppressive drug regime.

5.2.4.1 Difference in proportion of TTV/TLMV bound to IgG and free TTV/TLMV in lung transplant recipients

Having already established the titre of TTV/TLMV changes over time in the serum of transplant recipients, the antibody response to the virus in the same two immunosuppressed transplant patients was investigated by comparing the amount of virus circulating freely in serum with the amount of virus complexed with IgG using the protein G capture method (Figure 5.2.19 to Figure 5.2.22).

The relationship between the titre of TTV/TLMV complexed with IgG and free virus was explored initially by plotting the number of PCR positive dilutions for both fraction of each sample as shown in Figure 5.2.23 and Figure 5.2.24. They demonstrate that the relative proportion of free virus and complexed virus changes over time.

The disparity between the two portions of serum was also evaluated by calculating the difference between free TTV/TLMV and TTV/TLMV bound to IgG (free virus - bound virus) and these values were plotted with the titre of TTV/TLMV per ml of serum (Figure 5.2.25 and Figure 5.2.26). These figures show that apart from sample nine from patient H21 (day 588 post transplant which corresponds to a sharp decrease in TTV/TLMV titre), there always appears to be more free virus in serum that virus complexed to IgG. The sequential serum samples taken from patient H10 show the level of free virus increased to peak at day 251 post transplant whereas the difference in PCR positive dilutions from the portions of serum from H21 seems to correspond to changes in virus titre. Spearman's test for significance was carried out to investigate a correlation between the proportion of bound and unbound virus and the titre of TTV/TLMV (H10 p-value = 0.225, H21 $p<0.001$) indicating there is a relationship between these two factors in patient H21. Linear regression was used to test for any association between the dose and type of immunosuppressive drugs and the proportion of bound and unbound virus. A significant p-value ($p = 0.047$) was obtained when the dose of azathioprine was examined in patient H21. It is possible that in this patient the dose of azathioprine in some way contributed to the change in proportion of free virus compared with virus complexed with IgG.

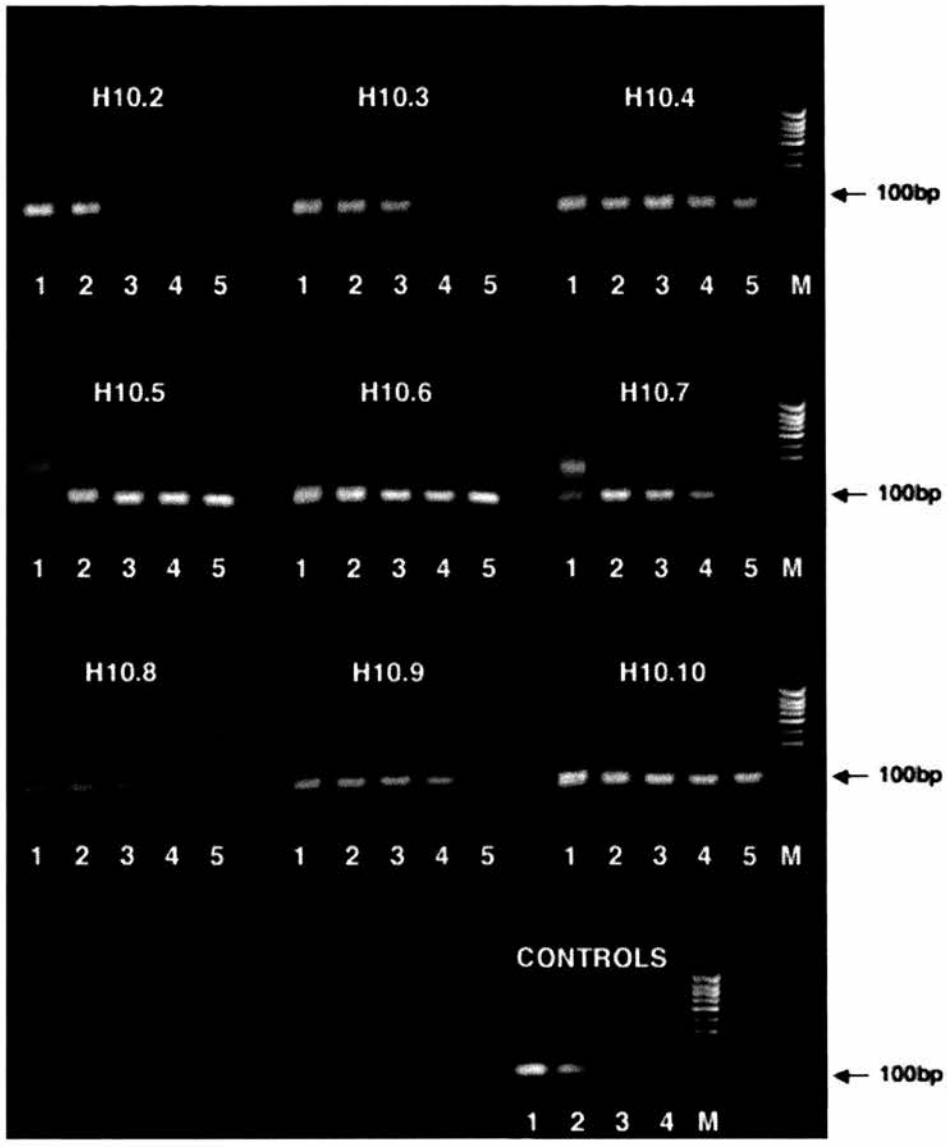

Figure 5.2.19 - UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from IgG depleted serum from lung transplant recipient H10. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: 2 μl DNA then 10 fold dilutions to Lane 5: $2x10^{-4}$ μl DNA); Lane M: 100bp DNA ladder. Controls, Lanes 1&2: Positive controls (minipools); Lanes 3&4: Negative controls (water).

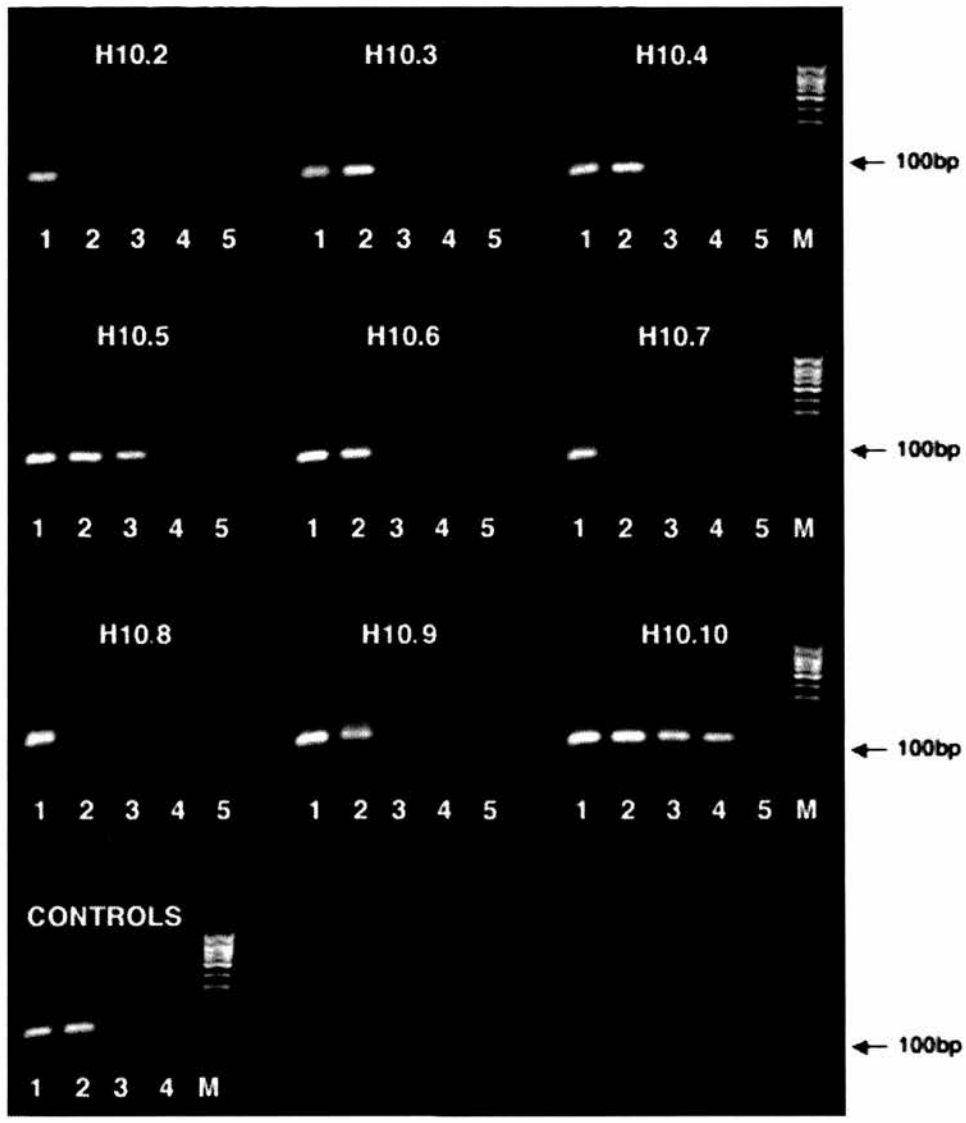

Figure 5.2.20 - UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from serum IgG isolated from lung transplant patient H10. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: 2 μl DNA then 10 fold dilutions to Lane 5: $2x10^{-4}$ μl DNA); Lane M: 100bp DNA ladder. Controls, Lanes 1&2: Positive controls (minipools); Lanes 3&4: Negative controls (water).

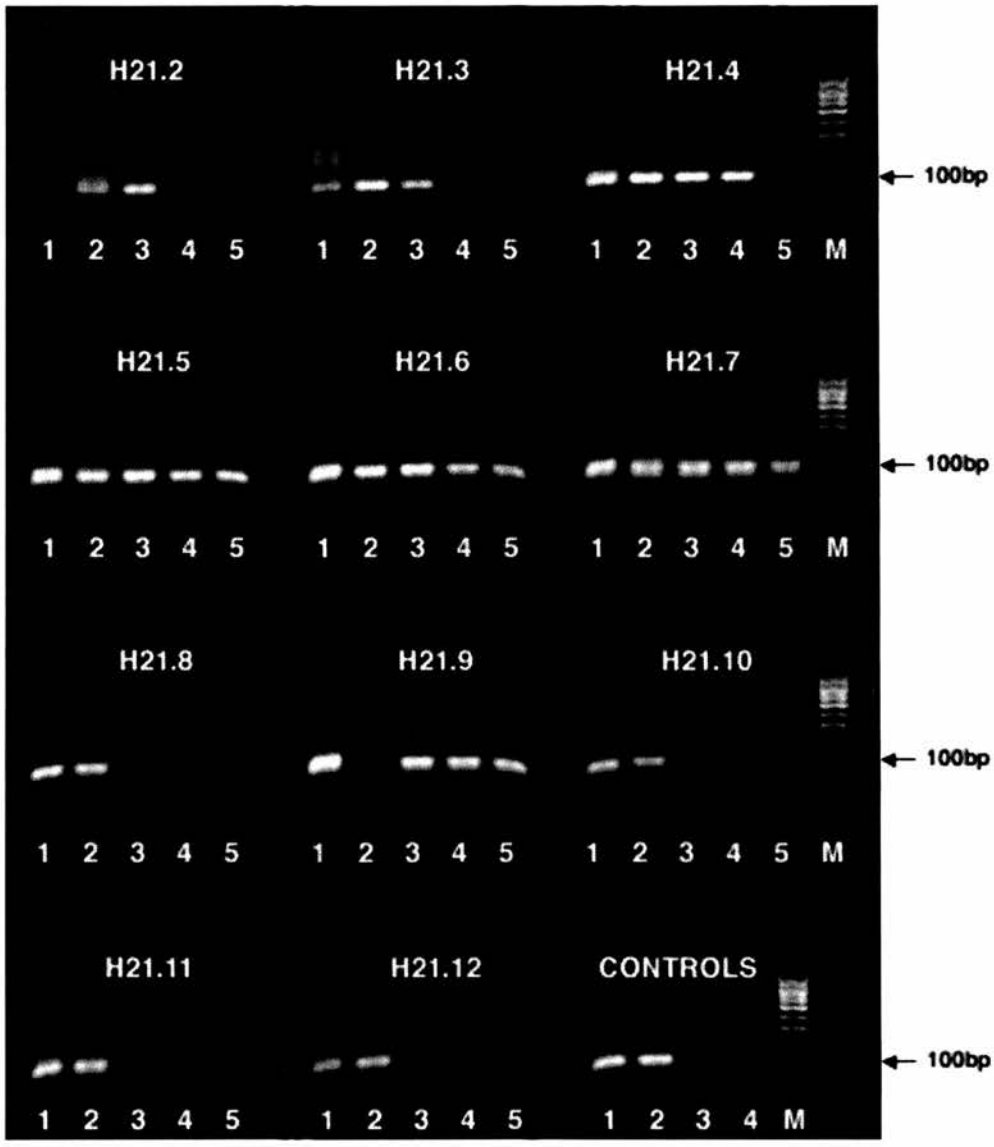

Figure 5.2.21 - UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from IgG depleted serum from lung transplant recipient H21. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: 2 μl DNA then 10 fold dilutions to Lane 5: $2x10^{-4}$ μl DNA); Lane M: 100bp DNA ladder. Controls, Lanes 1&2: Positive controls (minipools); Lanes 3&4: Negative controls (water).

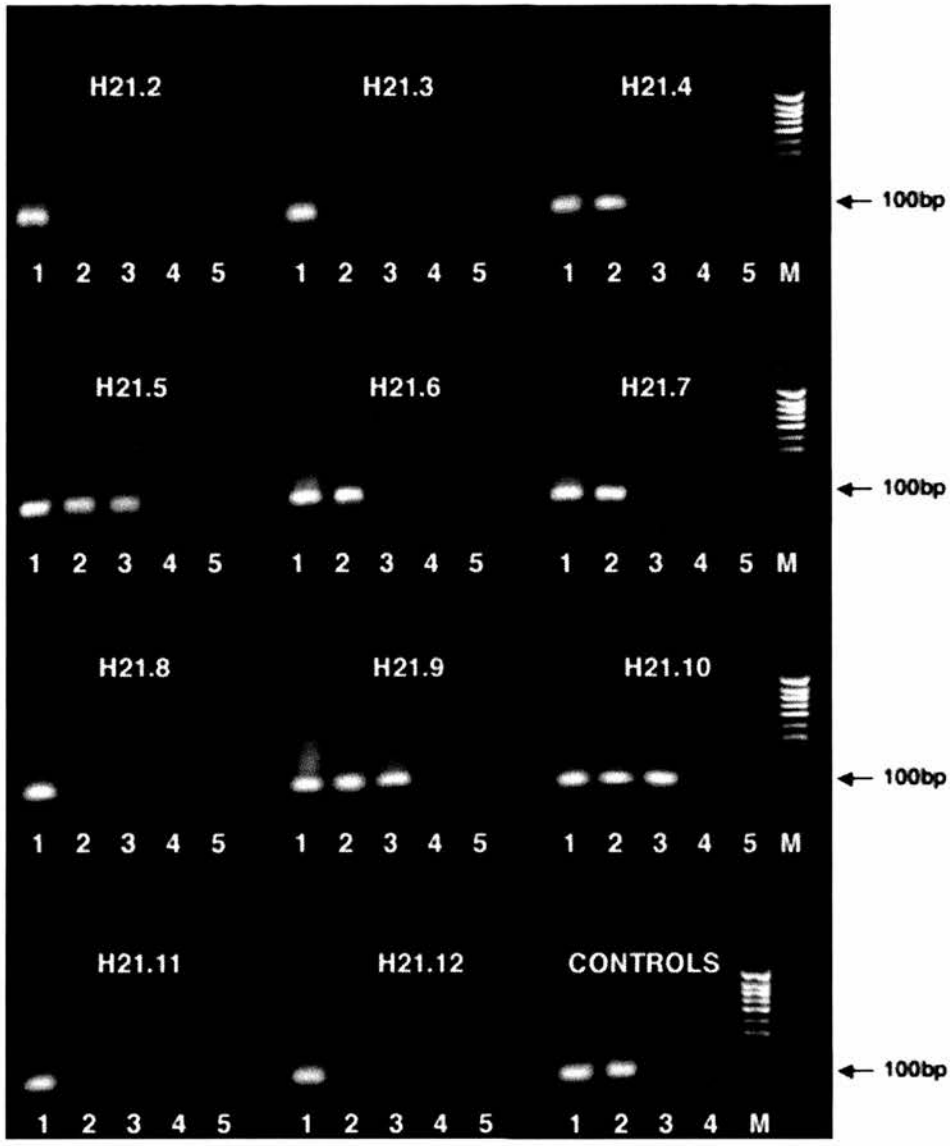

Figure 5.2.22 - UTR PCR for TTV/TLMV on sequential dilutions of DNA extracted from serum IgG isolated from lung transplant patient H21. Sample numbers are shown above each row of the agarose gel. Lanes 1-5: Dilutions of DNA in water added to the primary PCR reaction (Lane 1: 2 μl DNA then 10 fold dilutions to Lane 5: $2x10^{-4}$ μl DNA); Lane M: 100bp DNA ladder. Controls, Lanes 1&2: Positive controls (minipools); Lanes 3&4: Negative controls (water).

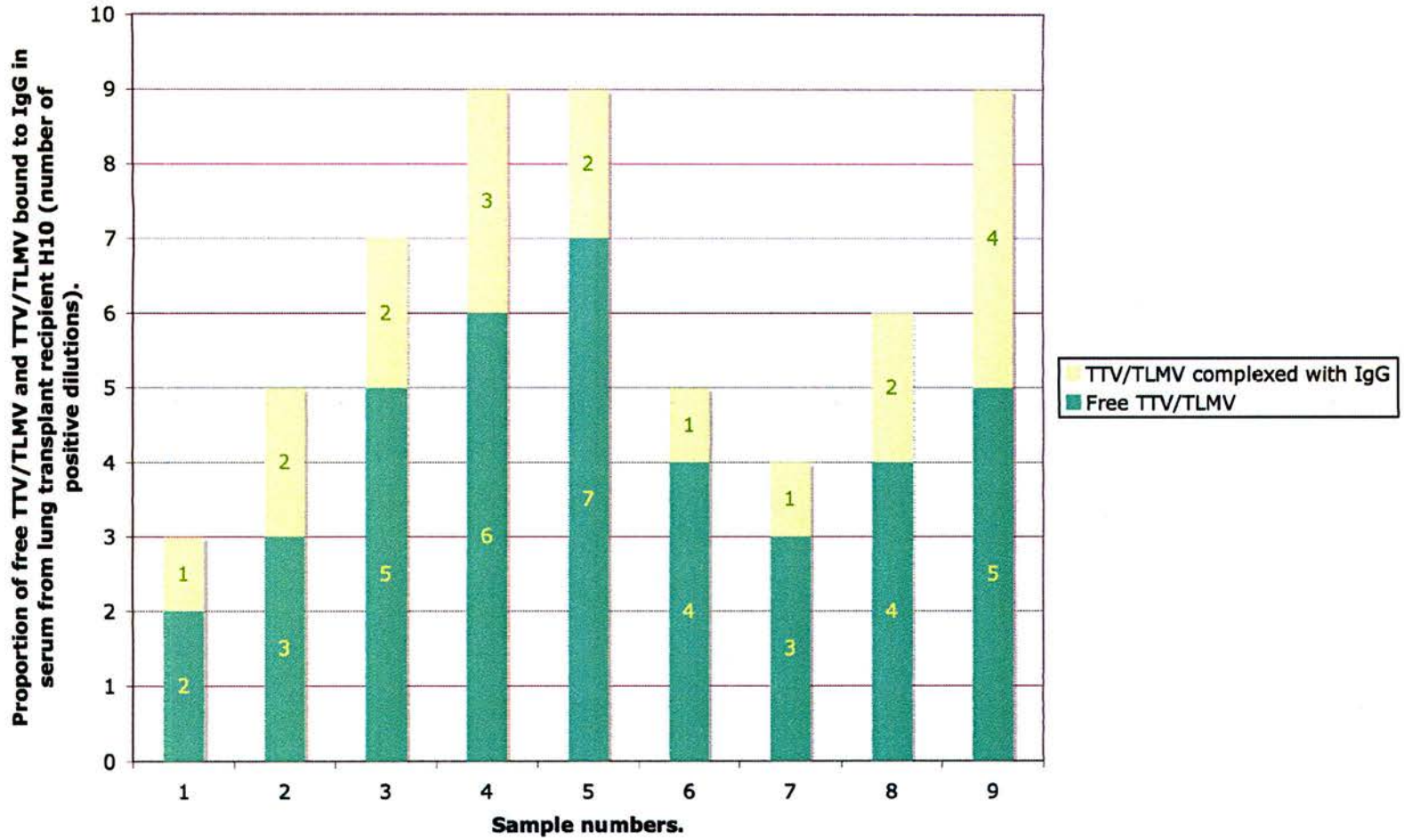

Figure 5.2.23 - Graph depicting the proportion of TTV/TLMV bound to IgG compared with the proportion of free virus in serum from lung transplant recipient H10. The number of positive dilutions of complexed and free virus as indicated by limiting dilution PCR using UTR primers are shown as digits on the graph.

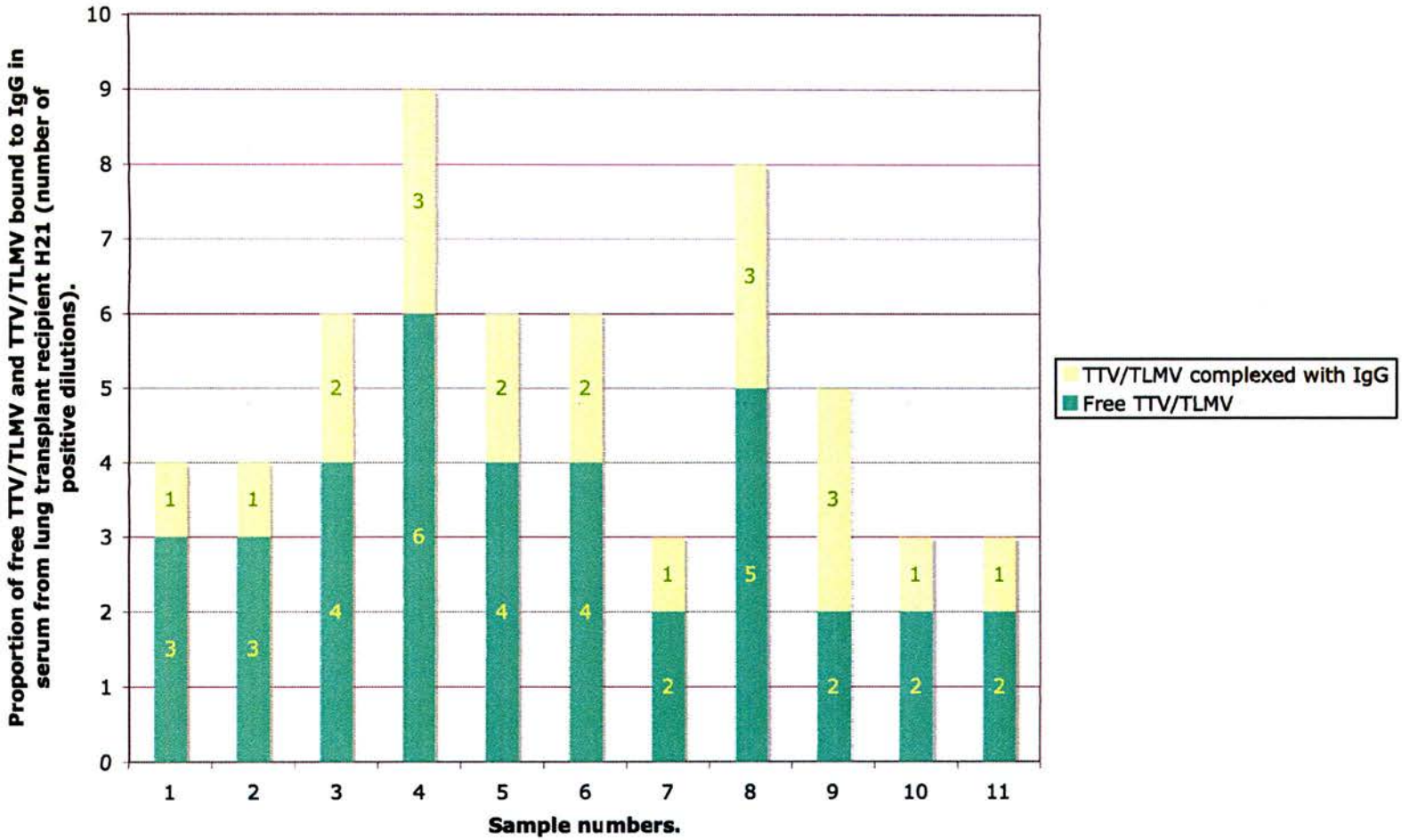

Figure 5.2.24 - Graph depicting the proportion of TTV/TLMV bound to IgG compared with the proportion of free virus in serum from lung transplant recipient H21. The number of positive dilutions of complexed and free virus as indicated by limiting dilution PCR using UTR primers are shown as digits on the graph.

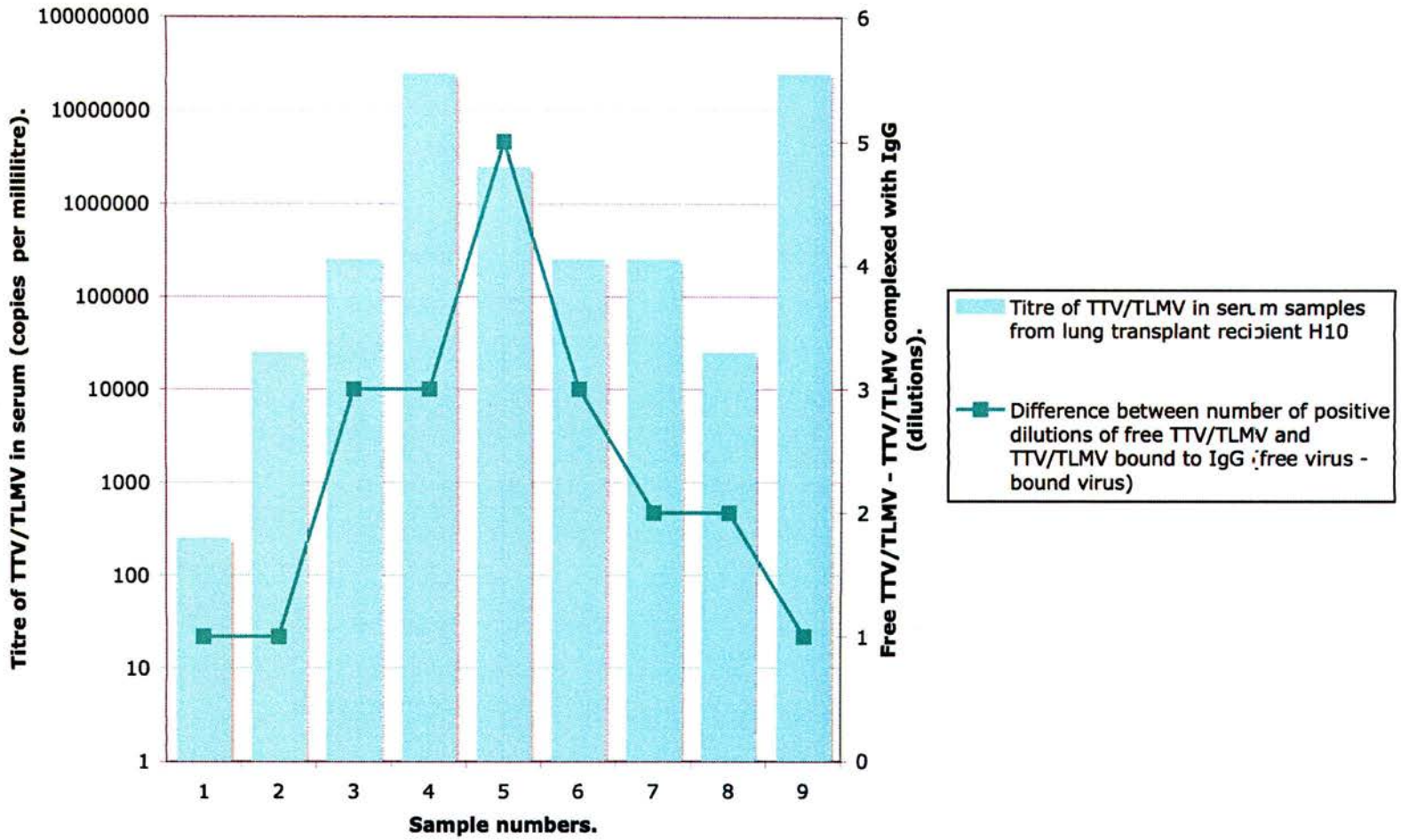

Figure 5.2.25 - Number of copies of TTV/TLMV per ml of serum taken over a period of 524 days post transplant from lung transplant patient H10 with the difference in proportion of free TTV/TLMV and TTV/TLMV bound with IgG over time.

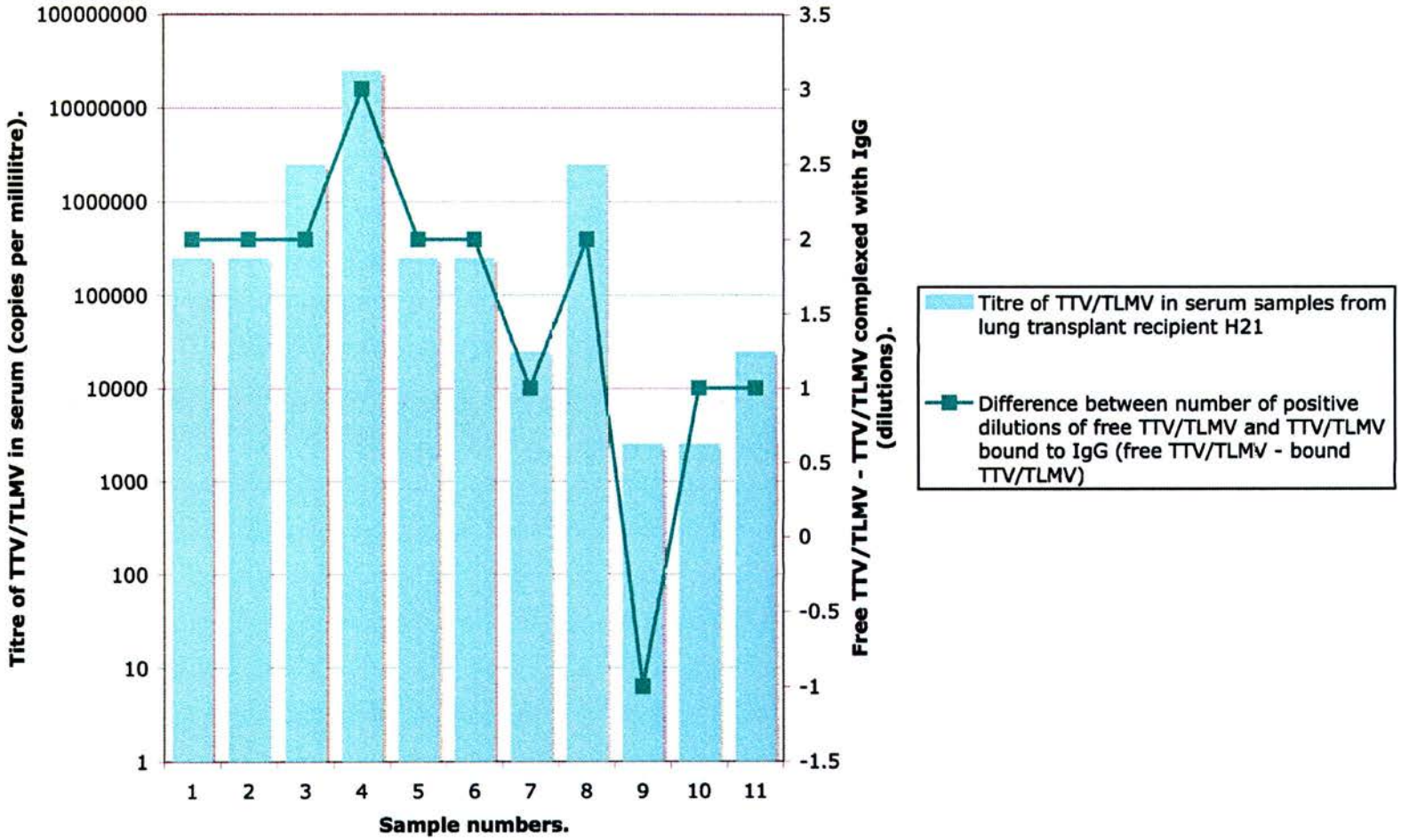

Figure 5.2.26 - Number of copies of TTV/TLMV per ml of serum taken over a period of 917 days post transplant from lung transplant patient H21 with the difference in proportion of free TTV/TLMV and TTV/TLMV bound with IgG over time.

5.3 Discussion

Having already shown that TTV/TLMV is present in higher titres in the bone marrow and spleen of HIV positive individuals who have progressed to AIDS compared with tissues taken from individuals without HIV infection, the aim of this chapter was to clarify the dynamics of TTV/TLMV infection in sera taken from immunosuppressed and immunocompetent patients. Sera from HIV positive patients were analysed for the presence of TTV/TLMV and the titres compared with those found in patients with hepatitis C or hepatitis B viruses, neither of which are associated with overt immunosuppression. Sera from healthy blood donors negative for HIV, HCV and HBV were used as a control group.

When ten samples from each of the four groups were analysed, 100% of the sera from individuals infected with a second viral marker (HIV/HBV/HCV) were PCR positive for TTV/TLMV however only 60% of the healthy group were TTV/TLMV PCR positive. Anellovirus infection in the remaining 40% of the HIV/HCV/HBV negative group cannot be ruled out however, it may be the virus is present at levels below the detection limit of the assay. Epidemiological studies have investigated the titre of TTV within a number of populations and found that the incidence of TTV was decreased in low risk populations such as blood donors compared with populations at risk of parenteral exposure [Gallian et al., 1999; Maggi et al., 1999]. Touinssi suggested that the reported increased prevalence of TTV was the result of elevated viraemia in high risk populations which increased the rate of detection of TTV [Touinssi et al., 2001]. We found a statistically significant increase in TTV/TLMV titre in the sera of HIV positive patients compared with the HCV and HBV positive groups and the HIV/HBV/HCV negative groups. This finding corroborates the results of the previous chapter which described increased titres of TTV/TLMV in tissues of HIV infected patients and an inverse correlation between CD4+ lymphocyte count and TTV/TLMV titre, again suggesting this is the result of the immunosuppression associated with HIV as opposed to the concomitant viral infection. Although the differences between the control group and the HCV and HBV positive group were not significant, the p-values (0.063 for HBV group and 0.07 for HCV group) suggested a trend towards higher TTV/TLMV titres in these groups. HCV, HBV and the Anelloviruses share parenteral transmission routes and there has been evidence that this route of transmission is associated with elevated TTV titres [Zehender et al., 2001]. It should be noted that the HIV, HCV and HBV positive samples were collected from blood donors for confirmatory testing. As such, the individuals were unaware of their infection and were not undergoing

any treatment that might affect TTV/TLMV titres, for example interferon alpha taken for HCV infection, which has been shown to decrease the serum titres of TTV in some patients.

The incidence of TTV was found to be increased in the sera of recipients of renal transplants, as determined by N22 PCR [Szladek et al., 2003] and increased titre of TTV in conjunction with immunosuppressant medication following kidney transplant [Moen et al., 2003] has also been recorded. In this study we semi-quantitated the TTV/TLMV present in the sera of recipients of lung, heart or heart and lung transplants. All 20 of the samples were PCR positive for Anelloviruses, with 90% of samples having a titre of 2500 copies of TTV/TLMV per ml. Statistical analysis revealed the titre of TTV/TLMV in transplant samples was significantly higher than titres in sera from HCV or HBV positive individuals and healthy blood donors however, the transplant samples had a significantly lower titre than the HIV positive samples. It is hypothesised that the drug induced immunosuppression needed to prevent the rejection of organs following transplant allows for an increase in the titre of TTV/TLMV. Although this increased TTV/TLMV titre in transplant recipients has not been associated with any pathology, an increase in titre or the reactivation of latent viruses is recognised as having a detrimental effect on the recipients of solid organ transplants. Of note are the Herpesviruses: Cytomegalovirus (CMV) which can effect any organ system, enhance immunosuppression and can cause rejection of the transplanted organ, Herpes simplex virus (HSV) infections post transplant sometimes result in ulcerative oesophagitis, tracheobronchitis and pneumonia, and Epstein-Barr virus (EBV) which can initiate post-transplant lymphoproliferative disorder (PTLD), a disease of uncontrolled proliferation of B lymphocytes which may form tumour masses [Slifkin, 2004].

There has yet to be conclusive evidence of TTV in transplant recipients resulting in hepatitis or liver injury [Kanda et al., 1999; Michitaka et al., 2000; Shang et al., 2000; Usta et al., 2002] however, one recipient of a bone marrow transplant developed hepatic injury corresponding to increased ALT levels which showed correlation to TTV titre. Liver biopsy reported pathology consistent with viral hepatitis and sequence analysis found a new genotype of TTV genetically distinct from TTV isolated before transplant. The authors concluded this novel TTV genotype may be the cause of the viral hepatitis [Kanda et al., 1999].

An increase in viral titres in response to immunosuppressive medication is not the only possible explanation for the high TTV/TLMV viral load seen in transplant recipients. Peri-transplant blood transfusions or the transplanted organ itself may introduce novel genotypes into the transplant recipient [Shang et al., 2000].

Immunosuppressive drugs are given to patients post-transplant to prevent the rejection of the transplanted organ. The transplant recipients used in this study received cyclosporine A, a calcineurin inhibitor which prevents the activation of interleukin-2 transcriptions and thus decreases the function of the cytotoxic T cells; azathioprine, a prodrug which converts *in vivo* to 6-mercaptopurine. This acts as an antagonist to prevent the synthesis of purines needed for the proliferation of lymphocytes. Prednisolone and methylprednisolone are corticosteroids which target several stages of immune stimulation inhibiting antigen presentation, cytokine production and the proliferation of lymphocytes [Offermann, 2004]. Linear regression found no evidence of association between TTV/TLMV titre and drug dose for prednisolone, methlyprednisolone and azathioprine however, although there did appear to be a trend towards increasing virus titre in response to increasing cyclosporine A dose, the findings were not significant.

The immune responses to TTV and TLMV that allow the development of chronic, and possibly life-long, viral infections are largely unknown. TTV complexed with IgG has been isolated from the serum of HIV positive patients and visualised using immunogold electron microscopy [Itoh et al., 2000]. This was one of the first pieces of evidence to suggest that the immune system was activated in response to infection. Antibody studies have also been published using TTV particles and portions of the ORF1 protein as antigen [Ott et al., 2000].

The first antibody secreted by the body in response to viral challenge is IgM. This is transient and the levels in the blood decline rapidly. A study has found anti-TTV IgM in two patients who developed post transfusion non A-G hepatitis coupled with acute TTV infection post transfusion [Tsuda et al., 2001] as well as in healthy adults [Ott et al., 2000] however, in the latter group, the IgM response was found to persist for years in conjunction with circulating TTV.

IgG is the most abundant antibody, able to enter tissue as well as being present in the circulation. It defends against viral infection both by activation of complement and opsonisation of the virus for phagocytosis. Anti-TTV IgG has been detected for prolonged

periods of time in a TTV DNA positive individual (detection of antibody was still possible when the study period ended at 4.4 years), appearing after IgM disappeared from circulation [Tsuda et al., 2001]. Ott and colleagues reported the isolation of IgG antibodies in the sera of healthy adults but at a considerably lower frequency than the detection of TTV DNA (10% IgG positive compared with 86% TTV DNA positive) [Ott et al., 2000]. The increased TTV DNA titre in immunosuppressed individuals reported in this study suggested a less robust immune response than in immunocompetent adults. To explore this theory, the titre of free TTV/TLMV was compared with TTV/TLMV complexed with IgG. This showed that the immunosuppressed individuals (HIV positive and transplant recipients) always had free virus titres which were greater than or equal to the titre of virus bound to IgG. This was also seen in all but one of the sequential samples analysed from two transplant recipients. Ten % of HCV positive, 20% of HBV positive samples and 40% of HIV/HCV/HBV negative samples had a higher titre of virus complexed with IgG compared with free virus. This suggests that in patients with immunosuppression the increased TTV/TLMV titre could be a result of a decreased IgG response to the viruses. Evidence that the stimulated PBMCs, but not unstimulated PBMCs were capable of supporting TTV replication [Maggi et al., 2001b] could imply at least some genotypes of TTV are able to infect cells of the immune system and that co-infection with other viruses and the resulting immune stimulation may produce the increased viral titres reported in this, and other, studies. On the other hand, it has been suggested hypervariable regions of the TTV genome allow the circulation of quasispecies of the virus which may escape immune surveillance [Nishizawa et al., 1999] and that number of genogroups present in serum increased exponentially with the viral load [Maggi, 2005]. Unfortunately, conclusions cannot yet be drawn about the possibility that increased viral titres increase the pathogenicity of the virus.

The TTV/TLMV titres of two transplant recipients (H10 and H21) fluctuated over the 1.5 and 2.5 year interval studied. Both patients virus titres increased steadily to peak at time point four however, this did not correspond to any change in immunosuppressive medication. Moen and colleagues described increases in the titres of TTV and TLMV 50-100 days after kidney transplant that corroborates the data obtained in this study.

The data presented in this chapter have given further insight into the dynamics of TTV/TLMV infection in healthy and immunosuppressed individuals. Sera from HIV positive patients had a titre of TTV/TLMV significantly higher that all of the other groups

studied with the majority of the virus circulating without forming complexes with IgG. Sera from transplant recipients also had significantly elevated Anellovirus titre which, like the virus seen in the HIV positive group, circulated as free virus. On the other hand, sera from healthy individuals had low titres of TTV/TLMV present in the serum which was predominately found circulating in complexed with IgG.

Chapter 6

Investigation of TTV/TLMV-like Viruses in Non-Human Primates and Farm Animals

6 INVESTIGATION OF TTV/TLMV-LIKE VIRUSES IN NON-HUMAN PRIMATES AND FARM ANIMALS

6.1 Introduction

Non-human primates, especially chimpanzees, have been used to elucidate the routes of transmission of all the hepatitis causing viruses. This frequently involved inoculating the chimpanzee with sera from a human either infected with the hepatitis virus of interest or suffering from cryptogenic non A-E hepatitis. The ubiquitous nature of TTV and TLMV has resulted in the inadvertent transmission of these viruses to chimpanzees. It was found not only that chimpanzees could be infected with human forms of this virus but that the chronic nature of the infection in chimpanzees was similar to infections observed in humans [Mushahwar et al., 1999].

Investigations of wild caught chimpanzees have established that they harbour a species-specific TTV-like virus similar to, but distinct from, human TTV [Barnett et al., 2004; Verschoor et al., 1999] as well as human TTV, which may be the result of the increasing interaction wild chimpanzees have with humans as they encroach on their territory [Barnett et al., 2004]. TTV-like viruses have also been isolated from gibbons and rhesus monkeys [Luo et al., 2000; Nopporpanth et al., 2001].

Using primers from the N22 region of the genome, Leary detected TTV in chickens, pigs, cows and sheep with high identity to human sequences (79-99% at the nucleotide level) [Leary et al., 1999]. Since it has already been shown that chimpanzees could be infected with human TTV, TTV present in domesticated farm animals that was not genetically distinct from human TTV could be contributing to the ubiquitous nature of the virus within human populations or could have been transmitted from their human keepers.

This chapter discusses the investigation of sera from a number of non-human primates for the presence of TTV/TLMV-like viruses. It also attempts to clarify the presence of TTV-like viruses in domesticated farm animals. Identification of these homologues will not only give us insight into the origin of these viruses and their evolution but may also provide answers to outstanding questions as the exact method of replication and the mechanism of persistence of TTV/TLMV in humans.

6.2 Results

6.2.1 Prevalence of TTV-like viruses in non-human primates

To investigate the possibility that non-human primates harbour TTV or TTV-like viruses, serum from a number of non-human primates species were analysed. Nucleic acids were extracted from the sera of 13 drills (*Mandrillus leucophaeus leucophaeus*), two mandrills (*Mandrillus sphinx*), four cherry-capped mangabeys (*Cercocebus torquatus*), five chimpanzees (*Pan troglodytes troglodytes* and *Pan troglodytes vellerosos*), 19 gibbons (*Hylobates agilis, Hylobates lar, Hylobates muelleri, Hylobates moloch* and *Hylobates gabriellae*), 14 Bornean orangutans (*Pongo pygmaeus pygmaeus*), and one Western Lowland gorilla (*Gorilla gorilla gorilla*) and analysed for the presence of TTV and TLMV using PCR with primers situated in the UTR region of the genome. It was hoped that the conservation within this region observed when human sequences were analysed would extend to any TTV homologues infecting apes and African monkey species. A representative agarose gel is shown in Figure 6.2.1.

Figure 6.2.1 – PCR products from nucleic acid extracted from non-human primate species and amplified using the UTR hemi nested primer set CVOS/CVOA and CVOS/CVIA. Lane 1: negative control (water); Lanes 2-14: secondary PCR product from samples D1-3, D5 & 6 and L1-8 (Drills); Lanes 15-17; secondary PCR product from chimpanzee samples L20, L22 & L24; Lane 18; positive control (minipool); Lane 19: negative control (water).

It was found that not only did these animals harbour a virus in their serum which could be amplified using primers designed for human TTV, but the prevalence of these viruses were comparable with the level of infection seen in humans, as shown in Table 6.2.1.

Table 6.2.1 – Table of non-human primate samples tested for the presence of TTV and TLMV. [1] Drill rehabilitation and breeding centre. Nigeria. [2] Limbe wildlife centre, Limbe, Cameroon. [3] Pingtung rescue centre, Pingtung University, Taiwan. [4] Edinburgh Zoo, Scotland.

Species	Identifier	Subspecies	Location of primate	UTR PCR positive/negative (+/-)
Drill	D1	Mandrillus leucophaeus leucophaeus	Nigeria[1]	+
Drill	D2	Mandrillus leucophaeus leucophaeus	Nigeria[1]	+
Drill	D3	Mandrillus leucophaeus leucophaeus	Nigeria[1]	+
Drill	D5	Mandrillus leucophaeus leucophaeus	Nigeria[1]	+
Drill	D6	Mandrillus leucophaeus leucophaeus	Nigeria[1]	+
Drill	L1	Mandrillus leucophaeus leucophaeus	Cameroon[2]	+
Drill	L2	Mandrillus leucophaeus leucophaeus	Cameroon[2]	+
Drill	L3	Mandrillus leucophaeus leucophaeus	Cameroon[2]	-
Drill	L4	Mandrillus leucophaeus leucophaeus	Cameroon[2]	+

Drill	L5	Mandrillus leucophaeus leucophaeus	Cameroon[2]	+
Drill	L6	Mandrillus leucophaeus leucophaeus	Cameroon[2]	+
Drill	L7	Mandrillus leucophaeus leucophaeus	Cameroon[2]	+
Drill	L8	Mandrillus leucophaeus leucophaeus	Cameroon[2]	+
Mandrill	L11	Mandrillus sphinx	Cameroon[2]	+
Mandrill	L12	Mandrillus sphinx	Cameroon[2]	+
C.C. Mangabey	L13	Cercocebus torquatus	Cameroon[2]	+
C.C. Mangabey	L14	Cercocebus torquatus	Cameroon[2]	+
C.C. Mangabey	L15	Cercocebus torquatus	Cameroon[2]	+
C.C. Mangabey	L16	Cercocebus torquatus	Cameroon[2]	+
Chimpanzee	L17	Pan troglodytes troglodytes	Cameroon[2]	+
Chimpanzee	L18	Pan troglodytes vellerosos	Cameroon[2]	+
Chimpanzee	L20	Pan troglodytes vellerosos	Cameroon[2]	+

Chimpanzee	L22	Pan troglodytes troglodytes	Cameroon[2]	+
Chimpanzee	L24	Pan troglodytes troglodytes	Cameroon[2]	-
Gibbon	G2	Hylobates lar	Taiwan[3]	+
Gibbon	G3	Hylobates gabriellae	Taiwan[3]	+
Gibbon	G4	Hylobates muelleri	Taiwan[3]	+
Gibbon	G5	Hylobates agilis	Taiwan[3]	+
Gibbon	G6	Hylobates gabriellae	Taiwan[3]	-
Gibbon	G7	Hylobates gabriellae	Taiwan[3]	+
Gibbon	G8	Hylobates muelleri	Taiwan[3]	-
Gibbon	G9	Hylobates lar	Taiwan[3]	+
Gibbon	G10	Hylobates agilis	Taiwan[3]	-
Gibbon	G11	Hylobates agilis	Taiwan[3]	+
Gibbon	G12	Hylobates gabriellae	Taiwan[3]	-

Gibbon	G13	Hylobates moloch	Taiwan[3]	+
Gibbon	G14	Hylobates muelleri	Taiwan[3]	-
Gibbon	G15	Hylobates muelleri	Taiwan[3]	+
Gibbon	G16	Hylobates moloch	Taiwan[3]	+
Gibbon	G17	Hylobates agilis	Taiwan[3]	+
Gibbon	G18	Hylobates moloch	Taiwan[3]	-
Gibbon	G19	Hylobates lar	Taiwan[3]	-
Gibbon	G20	Hylobates muelleri	Taiwan[3]	+
Orangutan	O1	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Orangutan	O2	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Orangutan	O3	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Orangutan	O4	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Orangutan	O5	Pongo pygmaeus pygmaeus	Taiwan[3]	+

Orangutan	O6	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Orangutan	O7	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Orangutan	O8	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Orangutan	O9	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Orangutan	O10	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Orangutan	O11	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Orangutan	O12	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Orangutan	O13	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Orangutan	O14	Pongo pygmaeus pygmaeus	Taiwan[3]	+
Gorilla	R1	Gorilla gorilla gorilla	Edinburgh[4]	+

For the non-human primate samples, 100% viraemia was detected in orangutans, cherry-capped mangabeys, mandrills and the single gorilla tested. Twelve of the 13 drills tested (92%), 12/19 gibbons (63%) and 4/5 chimpanzees (80%) also had detectable virus in their serum. Nucleic acid from the serum of all 58 non-human primate samples were also tested for the presence of a TTV-like virus using the N22 primers which amplify a portion of ORF1. None of the samples had virus detectable using this primer set.

6.2.2 Investigating primate TTV/TLMV-like viruses using real time PCR

To assess the heterogeneity of the viruses amplified from the non human primate samples, a selection of primary product from nucleic acid amplified using the UTR primers (from section 6.2) were subjected to real time PCR amplification on the Roche Lightcycler as previously described. A representative melting temperature profile is shown in Figure 6.2.2. It was found that although some of the viruses amplified from the same species had the same or very similar melting temperatures (L4 and L5, Figure 6.2.2) there were also viruses within the species group which displayed markedly different Tms, for example L2 and L4/L5 (Figure 6.2.2). Some of the sera also showed two melting peaks, indicating infection with a heterogeneous mixture of virus (G5, Figure 6.2.2), possibly analogous to the TTV/TLMV co-infections seen in humans.

6.2.3 Cloning and sequencing of virus from non-human primate sera

The PCR products from the amplification of virus from non-human primate samples were inserted into pGEM-T easy vector and transfected into *E.coli* cells. After plating up the transformed cells and growing them overnight, cells from a number of colonies containing viral DNA isolated from the non-human primate serum samples were added to a primary PCR using the UTR primers. A secondary PCR reaction was carried out on the Lightcycler to confirm the ligation and transfection reactions had been successful. In order to investigate further the distribution of melting temperatures, Tms from real time PCR analysis of cloned virus were plotted in Figure 6.2.3.

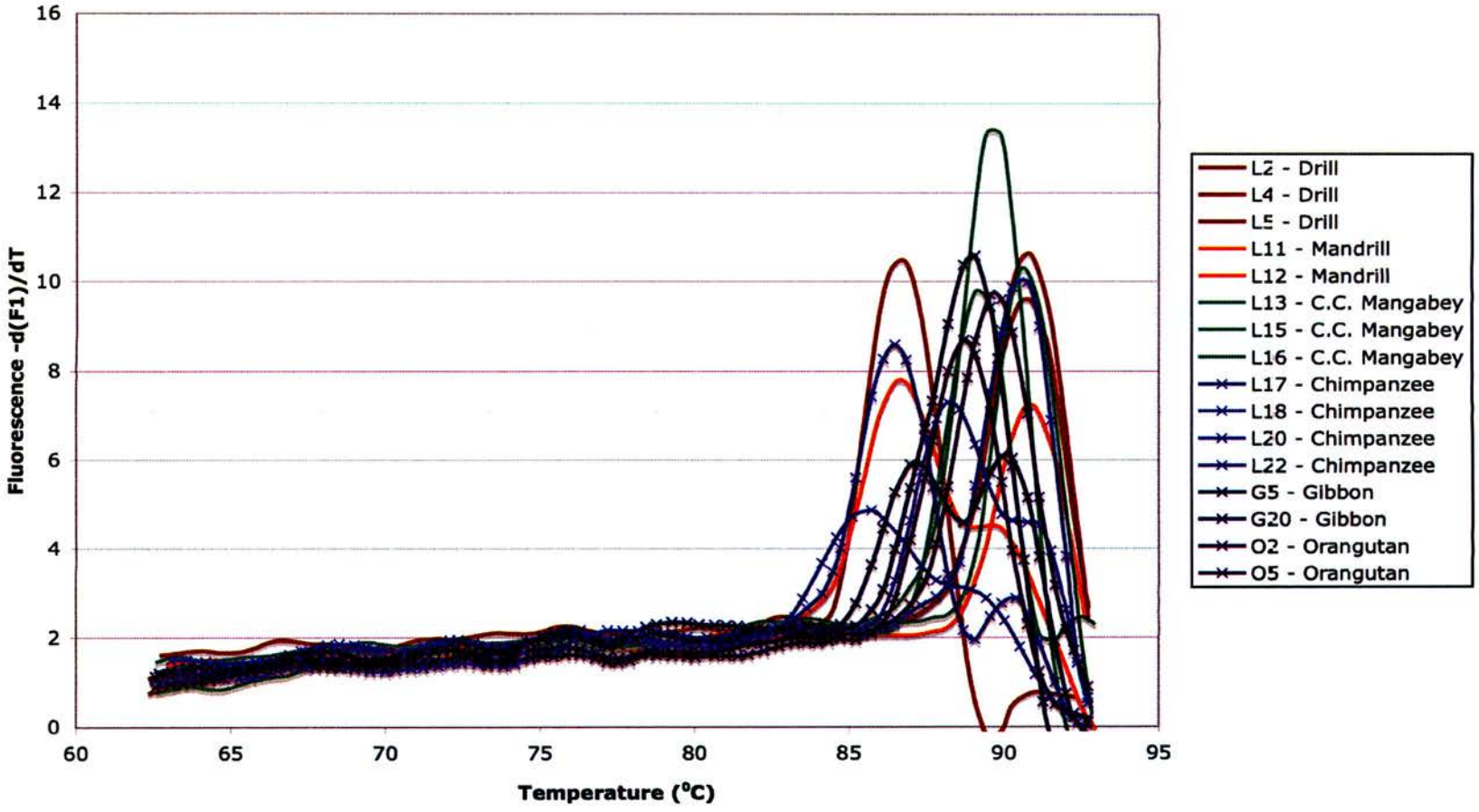

Figure 6.2.2 – Melting curve profiles of virus amplified from the serum of non-human primates and analyzed by real time PCR. A straight line (—) indicates an Old World monkey and apes are represented by ✕.

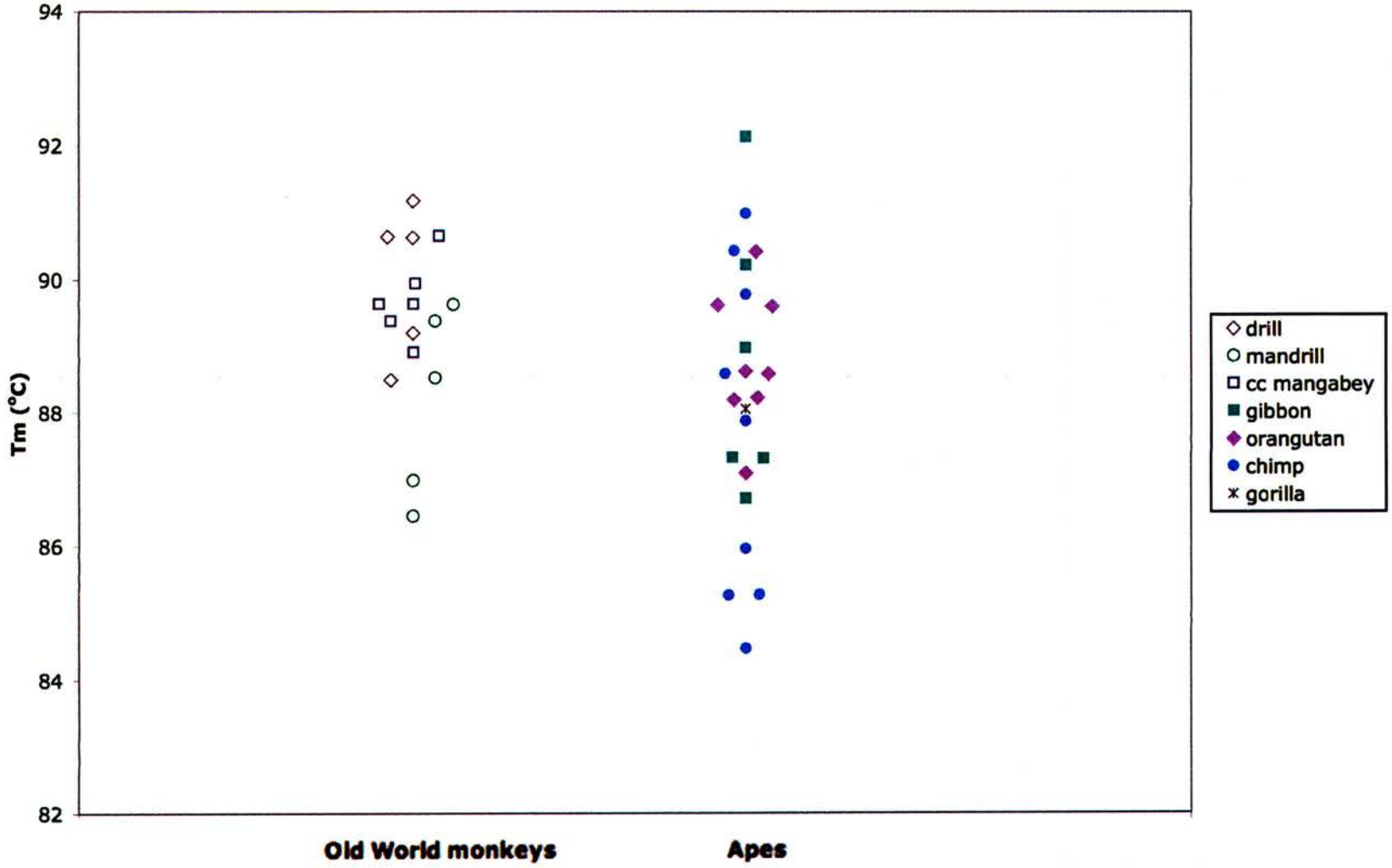

Figure 6.2.3 - Comparison of the distribution of melting temperatures of TTV/TLMV related viruses amplified from clones of virus present in the sera of Old World monkeys (unfilled symbols) and apes (filled symbols).

From the limited number of clones analysed, it appears that viruses from all the species investigated are as heterogeneous as TTV and TLMV isolated from human serum samples. In fact, the differences in Tms seen here could suggest that non-human primates may be infected with more than one type of Anellovirus that shares the partially conserved untranslated region. This pattern is most prominent when analysing the chimpanzee clones which appear to form two discrete groups, one with a high Tm (mean 89.5°C, median 89.7°C) and one with a low Tm (mean 85.7°C, median 85.6°C), which could correspond to a TTV-like and TLMV-like virus.

To test this theory, sequences were obtained from all of the primate groups and aligned using Simmonic software. These were then compared with human TTV and TLMV sequences and published sequences from chimpanzees as well as two sequences from long tailed macaques (*Macaca fasicularis*), and sequences from an owl monkey (*Aotus trivirgatus*), a cotton-topped tamarin (*Sanguinis oedipus*) and a tree shrew (*Tupaia belangeri chinesis*) (Figure 6.2.4).

Although human TTV and TLMV have regions of conserved sequences within the untranslated region, aligning the two viruses can be problematic because of the degree of sequence heterogeneity. This proved an issue during the alignment of the sequences shown in Figure 6.2.4, with sequences generated from both human and non-human primates showing such divergence that the alignment became arbitrary. To ensure a degree of consistency, the insertion of gaps was limited to enable alignment of the downstream primer to one gap between position 161 and 162.

Analysis of chimpanzee sequences revealed they showed either similarity to human TTV (PT(v)) or human TLMV (Pt(l)). Sequences comparable to human TTV exhibited a characteristic such as a conserved 5' region and are generally between three and six nucleotides longer in length than the prototype TTV sequence.

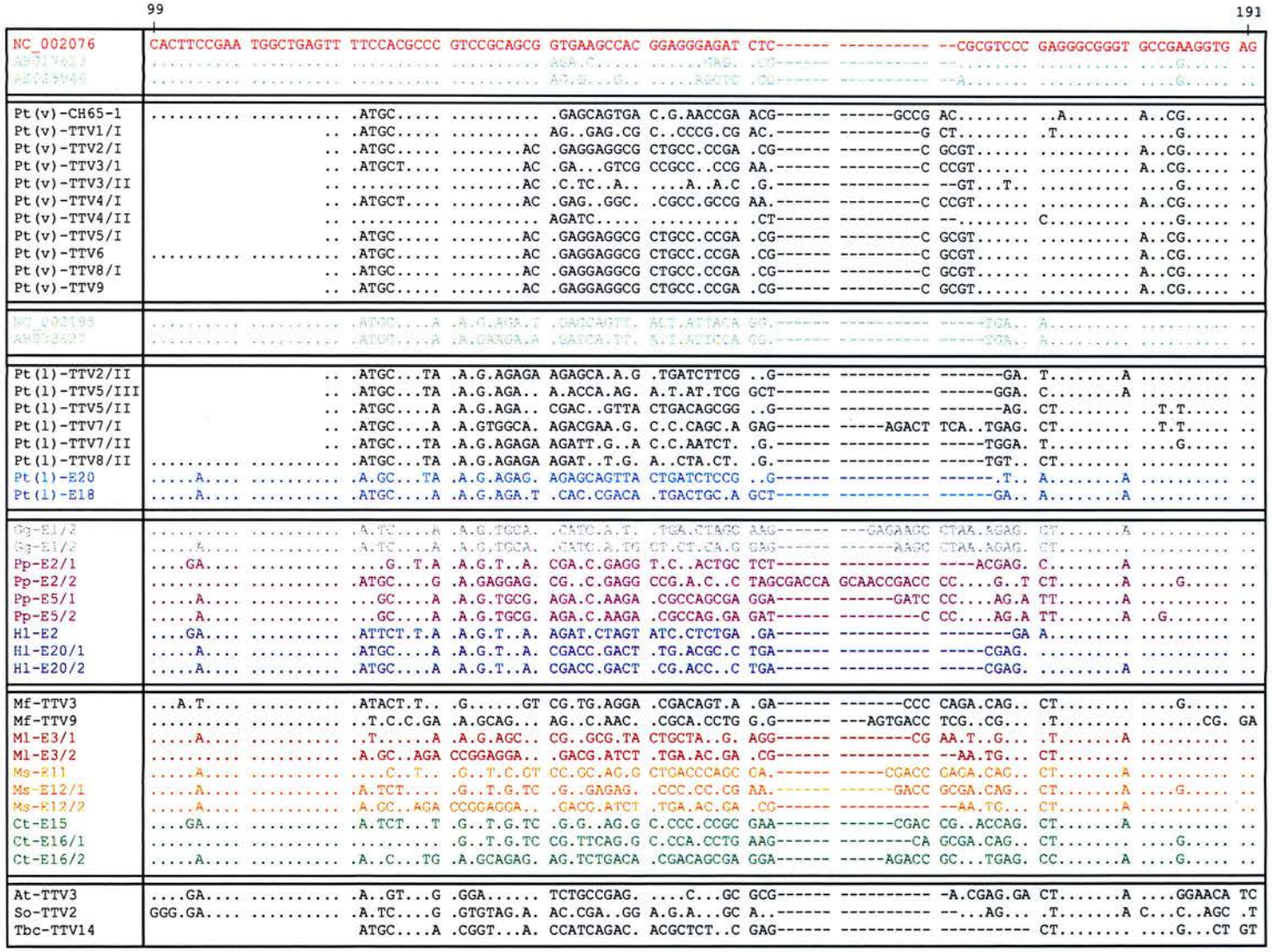
99
191
NC_002076
Pt(v)-CH65-1
Pt(v)-TTV1/I
Pt(v)-TTV2/I
Pt(v)-TTV3/1
Pt(v)-TTV3/II
Pt(v)-TTV4/I
Pt(v)-TTV4/II
Pt(v)-TTV5/I
Pt(v)-TTV6
Pt(v)-TTV8/I
Pt(v)-TTV9
Pt(l)-TTV2/II
Pt(l)-TTV5/III
Pt(l)-TTV5/II
Pt(l)-TTV7/I
Pt(l)-TTV7/II
Pt(l)-TTV8/II
Pt(l)-E20
Pt(l)-E18
Pp-E2/1
Pp-E2/2
Pp-E5/1
Pp-E5/2
Hl-E2
Hl-E20/1
Hl-E20/2
Mf-TTV3
Mf-TTV9
Ml-E3/1
Ml-E3/2
Ms-E11
Ms-E12/1
Ms-E12/2
Ct-E15
Ct-E16/1
Ct-E16/2
At-TTV3
So-TTV2
Tbc-TTV14

Figure 6.2.4 – Comparison of TTV and TLMV sequences from the untranslated region of virus isolated from humans and non-human primates. Prototype TTV sequence NC_002076 (human genotype 1)[Kamahora et al., 2000] has been used as a reference sequence with symbols used to depict the sequence identity with the prototype sequence; '.' :sequence identical to prototype TTV strain; '-' :gap introduced to preserve alignment of homologous nucleotide sites. Two other human TTV sequences are included in the alignment, AB017613 (genotype 11)[Okamoto et al., 1999b] and AB025946 (genotype 13)[Hijikata et al., 1999] as well as two human TLMV sequences; NC_002195 and AB038627 [Takahashi et al., 2000a]. Sequences of non-human primate origin are shown with the species as a prefix: Pt: *Pan troglodytes troglodytes* and *Pan troglodytes vellerosos* (chimpanzee); Gg; *Gorilla gorilla gorilla* (Western lowland gorilla); Pg: *Pongo pygmaeus pygmaeus* (Bornean orangutan); Hl: *Hylobates lar*, *Hylobates agilis*, *Hylobates muelleri*, *Hylobates moloch*, *Hylobates gabriellae* (gibbons); Mf: *Maccaca fasicularis* (long tailed macaque); Ms: *Mandrullus sphinx* (mandrill); Ml: *Mandrillus leucophaeus leucophaeus* (drill); Ct: *Cerocebus torquatus* (cherry capped mangabey); At: *Aotus trivirgatus* (owl monkey); So: *Sanguinis oedipus* (tamarin) and Tbc:*Tupaia belangeri chinesis* (tree shrew). Chimpanzee sequences have been assigned a "v" of "l" following the species prefix to indicate TTV-like or TLMV-like. Sequences obtained in this study from chimpanzee, gorilla, orangutan, gibbon, drill, mandrill and cherry-capped mangabey have the prefix E. If more than one sequence was obtained from the same sample, the clone number is shown as a suffix.

The PT(v) sequences do, however, appear to share the ATGC motif at positions 120-123 with both human TLMV and chimpanzee TLMV-like viruses. Pt(l) sequences have high degree of sequence conservation with human TLMV at the 5' end of the untranslated region but tend to be 1-2 nucleotides shorter than human TLMV and 3-5 nucleotides shorter than the prototype human TTV.

The sequences showed remarkable length polymorphisms. In this portion of the virus genome, human TLMV tends to be three nucleotides shorter than human TTV however the disparity in length between the primate sequences and prototype human TTV sequence (NC_002076) is far greater. The orangutan sequence Pp-E2/2 was found to be 18 nucleotides longer than the prototype human sequence while H1-E2, a sequence obtained from a gibbon was six nucleotides shorter. The mean length polymorphisms for all of the species sequences are summarised in Table 6.2.2. Both the chimpanzee TLMV-like sequences and the gibbons sequences were shorter than prototype human TTV while the rest of the non-human primates investigated had viral genomes with an untranslated regions that were longer than NC_002076.

Due to the length polymorphisms and heterogeneity of the primate samples, phylogenetic analysis of the region was thought to be inappropriate, as it relied on the assumption that homologous bases were aligned. In order to illustrate these data, a graphical representation was achieved by comparing each primate sample with sequences representative of the untranslated region of both TTV (n=58) and TLMV (n=50) (Figure 6.2.5). Also included were the human sequences obtained from human serum samples in chapter three (Figure 3.1.8).

The human sequences fell into two reasonably well defined clusters; one showing 20-30% sequence divergence from TTV but greater than 50% divergence from TLMV, the second 10-35% sequence divergence from TLMV and greater than 50% from TTV. This suggests that TLMV is in fact more divergent than TTV.

Species	Sequence prefix	Number of sequences	Mean difference in length from prototype sequence NC_002076 (nucleotides)	Range of differences (nucleotides)
Chimpanzee TTV-like	Pt(v)	11	+2.7	0,+6
Chimpanzee TLMV-like	Pt(l)	8	-2.6	-5,+7
Gorilla	Gg	2	+7.5	+6,+9
Orangutan	Pp	4	+6.2	-2,+18
Gibbon	Hl	3	-4	-6,-3
Macaque	Mf	2	+7	+5,+9
Drill	Ml	2	+2	0,+4
Mandrill	Ms	3	+6.5	0,+7
Cherry-capped mangabey	Ct	3	+5.6	+4,+7

Table 6.2.2 – Table showing the mean difference in length polymorphisms between primate species when compared with the prototype human TTV sequence NC_002076.

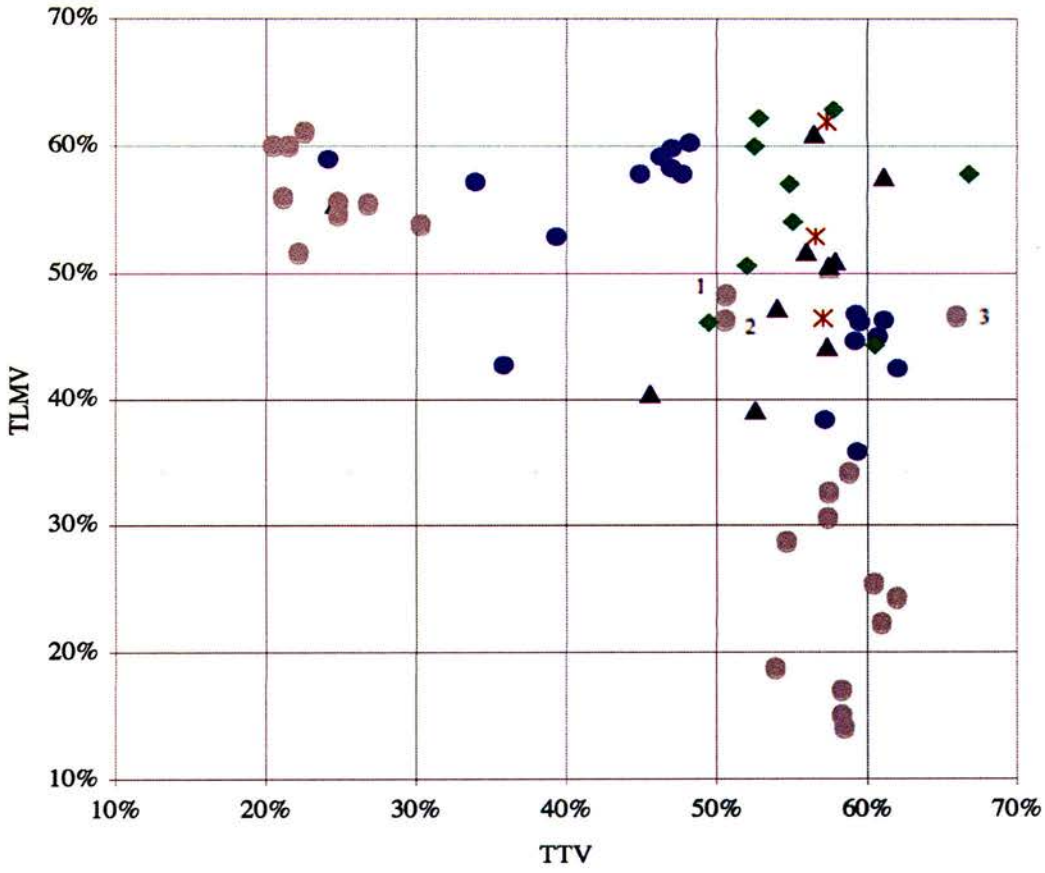

Figure 6.2.5 –Sequence divergence of human (Figure 3.1.8) and primate (Figure 6.2.4) TTV-related UTR sequences from representative sequences of TTV (n=58) and TLMV (n=50). Distances from TTV (x-axis) and TLMV (y-axis) sequences shown as percentage divergence. Symbols were used to indicate the species of origin of the UTR sequences: ● : human; ● : chimpanzee; ▲ : other apes; ◆ : Old World monkey species; ✱: New World primates and tree shrew. 1,2 and 3 denote human TLMV 1/1, 1/2 and 1/3 which do not appear to cluster with the other human samples.

Interestingly, human sequences TLMV 1/1, 1/2 and 1/3 do not group with the other two clusters of human samples. Both amplified with a TLMV specific primer, they show greater than 50% sequence divergence from TTV and 45% from TLMV. Sequence analysis of TLMV 1/1 and 1/2 (Figure 3.1.8) demonstrated an unusual six nucleotide deletion at position 162 as well as abnormally low Tm values of 83.7°C (Figure 3.1.9). The high percentage of sequence divergence could point towards this virus being a member of a third uncharacterised member of the Anellovirus genus.

As the alignment in Figure 6.2.4 shows, chimpanzee sequences appear to mimic the human TTV and TLMV relationship, grouping into two separate clusters: TTV-like sequences (n=ten) which showed 24-48% sequence divergence from TTV compared with greater than 50% divergence from TLMV, and TLMV-like chimpanzee sequences (n=eight) displaying 36-47% divergence from TLMV and greater than 50% from TTV.

Sequences from the other primate species group in the upper left quadrant of Figure 6.2.5, being approximately 40-70% divergent from human TLMV and 50-70% divergent from human TTV.

6.2.4 TTV-like viruses infecting farm animals

Leary and colleagues reportedly found TTV in chickens, pigs, cows and sheep using primers which amplified a portion of the heterogeneous ORF1 [Leary et al., 1999]. The virus from these animals was found to be "not genetically distinct from those (isolates) found in humans". To investigate the presence of TTV like viruses in the UK farm animal population, 20 cows, 20 sheep, 20 goats and 29 chickens were tested for the presence of TTV-like viruses in their serum using PCR which amplified a portion of the untranslated region as well as two methods targeting the ORF1 region. Of the 89 farm animal samples tested, none of them showed signs of infection with any TTV-like viruses.

6.3 Discussion

The conserved UTR region of the TTV and TLMV genome has been invaluable in the development of detection methods for these viruses. Alignment of limited TTV-like sequences from chimpanzees and a macaque [Inami et al., 2000; Okamoto et al., 2001b] suggested this region may also be conserved in any Anellovirus infecting non-human primates. Sera from a variety of apes and African monkey species were subjected to analysis by PCR using primers from this conserved region and it was found that high

frequency of primates (63-100%) were infected with a TTV/TLMV-like virus. Such high prevalence has been confirmed in other studies which found 83-100% of chimpanzees, Japanese macaques, red–bellied tamarins, cotton-top tamarins and douroucoulis were infected with TTV [Okamoto et al., 2000b]. TTV has also been isolated from tupaias, which share characteristics with both primates and insectivores [Okamoto et al., 2001b].

Melting curve analysis of the PCR products from the primates showed a much larger range of *Tm*s, especially in the African monkey group, compared with the same analysis of human TTV and TLMV. A selection of primates used in this study were subjected to sequence analysis and aligned with published TTV/TLMV sequences from humans as well as from chimpanzees, long tailed macaques, an owl monkey, a tamarin and a tupaia. None of the primate sequences from this study corresponded to known human genotypes of TTV and TLMV. Melting curve analysis of chimpanzee sequences frequently revealed two melting peaks leading to the hypothesis that chimpanzee sera harboured two Anelloviruses. The alignment confirmed that this was indeed the case and the UTR derived chimpanzee sequences could be classed as either TTV-like or TLMV-like. This is the first evidence of TLMV infection in non-human primates. Unlike the chimpanzee sequences, sequences derived from the other primate species showed no specific relationship with human TTV and TLMV. This implies that TTV and TLMV co-evolved with their hosts, and if both viruses have a common ancestor, the evolutionary split must have occurred at least seven million years ago before the speciation of humans and chimpanzees.

In this study, only a small portion of the relatively conserved untranslated region was sequenced however, full length sequences have also been reported. Like human TTV, chimpanzee TTV has partially overlapping open reading frames ORF1 and ORF2 however, comparison of the full length chimpanzee sequence with five full length sequences isolated from humans showed only 48-54% identity at the nucleotide level [Inami et al., 2000]. Four genogroups of chimpanzee TTV have been reported so far [Okamoto et al., 2000a], suggesting that chimpanzee viruses may be as heterogeneous as those found in humans. Chimpanzees infected with multiple genotypes of TTV have been described [Okamoto et al., 2000a; Romeo et al., 2000; Verschoor et al., 1999] as has a single case of a chimpanzee simultaneously infected with chimpanzee specific and human TTV [Okamoto et al., 2000a]. Phylogenetic analysis of examples of the four chimpanzee genogroups together with human TTV sequences revealed that the chimpanzee sequences

did not cluster together, but rather were found interspersed with the human genotypes [Okamoto et al., 2000a] as seen with HIV [Gao, 1999].

Although the full length genomes of TTV isolated from humans and chimpanzees are genetically divergent, the UTRs have a relatively high degree of sequence similarity [Inami et al., 2000] with a conserved GC-rich region and TATA box. Even the sequences isolated from Japanese macaque, tamarin and douroucoulis, all genetically dissimilar to the human sequences, share UTR nucleotide similarity of 57-72% with human UTR sequences. This high degree of conservation in such a heterogeneous virus suggests strong evolutionary pressure.

The high proportion of DNA shared between humans and chimpanzees, which make chimpanzees an excellent human model, also facilitates the spread of pathogens from one species to the other. As well as species-specific TTV-like viruses described in this and other studies [Verschoor et al., 1999], human TTV has also been isolated from non-human primates caught in the wild [Barnett et al., 2004]. This proves that chimpanzees can be infected with human TTV and corroborates a study outlining the successful transmission of human TTV purified from faeces to two chimpanzees [Tawara et al., 2000]. Antibodies to human TTV genotypes have also been isolated from chimpanzee serum without the presence of virus, indicating that chimpanzees are capable of resolving human infections [Okamoto et al., 2000a]. Although human TTV is not thought to be pathogenic *per se*, studies into the effects on chimpanzees are limited. One such study in which two chimpanzees were infected with human TTV reported mild biochemical changes including an increase of ALT in serum and limited hepatocellular ballooning observed during liver tissue biopsy [Tawara et al., 2000]. The outcome of chimpanzee specific TTV infection in humans is unknown however, as there has not been any reported instances of this [Barnett et al., 2004; Okamoto et al., 2000a]. HIV is thought to have originated from the Simian Immunodeficiency virus (SIV) and is the most well known example of a virus which is non-pathogenic in the host species jumping species and becoming a virulent virus with a high mortality rate [Gao, 1999].

The hypothesis that TTV and TLMV share a common ancestor and co-evolved with the host species is at odds with the discovery of TTV sequences similar to human TTV genotypes in domesticated farm animals [Leary et al., 1999]. Using two sets of primers derived from ORF1 of the human genome and one set from the relatively conserved UTR, the presence of TTV in sera from cows, sheep, goats and chickens was assessed. Even

when the conditions outlined in the original study were recreated, none of these animals were found to be infected with TTV. Given the divergence of the TTV isolated from the tupaia, and the appearance of mutations within the primer binding sites in TTV isolated from macaques and tamarins, it would seem safe to assume that any Anellovirus infecting mammals such as the domesticated farm animals used in this study would not be amplified using primers derived from human sequences. More recently, a TTV-like virus has been isolated from pigs, dogs and cats using a non-nested PCR with UTR primers. These sequences were phylogenetically closer to TTV isolated from the tupaia and lower order primates, such as the tamarin and douroucouli than human TTV and TLMV [Okamoto et al., 2002].

In summary, this study has confirmed the near universal infection of apes and African monkey species with an Anelloviruses. Sequence analysis of viruses isolated from chimpanzee sera recorded, for the first time, that the viruses tend to be either TTV-like or TLMV-like and melting curve analysis determined infection with a heterogeneous mixture of both virus types is not uncommon. A high degree of conservation of the UTR in sequences from non-human primates was observed, underlining the likely functional importance of this region of the genome. It has also been clarified that TTV capable of being amplified by the semi-nested PCR primers used throughout this thesis or primers specific to the heterogeneous ORF1 of human TTV are not present in domesticated farm animals.

Chapter 7

General Discussion

7 GENERAL DISCUSSION

Viral infections in humans most commonly result in one of three outcomes: 1) In relatively few instances, the virus infects the host and quickly causes the death of the infected individual. An example of this is Ebola virus, of the *Filoviridae* family, which has a 90% mortality rate. Although the reservoir for this virus has not yet been identified, there have been five outbreaks of the most pathogenic of these viruses, Ebola Zaire, in the last 10 years. 2) The most common scenario in human viral infections is the complete elimination of the virus from the host. Rhinovirus, a genus of the *Picornaviridae* family, is one of the viruses that cause the common cold and is the most common virus infection in humans. The virus replicates primarily in the respiratory tract and the infected cells secrete interleukins which attract neutrophils to the site of infection, leading to the elimination of infected cells by phagocytosis. The median length of rhinovirus infection is thought to be 11 days. 3) Viruses can also become latent following primary infection, resulting in the presence of the viral genome within the host but no overt disease. Following primary infection, Herpes simplex virus 1 (HSV-1) pervades the nerve ending and travels to the trigeminal ganglia where in maintains an extrachromosomal latent state for the life of the host, occasionally becoming reactivated in response to certain trigger factors. Latency in achieved by evasion of the immune system using a variety of mechanisms, however the most important of these is the production of the viral protein ICP47 which binds to the transporters associated with antigen processing and block the MHC class I restricted antigen processing, decreasing the production of CD8+ cytotoxic T lymphocytes. HSV-1 is also thought to be able to infect natural killer cells, thus inactivating the NK cells apoptotic activity.

The existence of viruses which can produce chronic, possibly life-long infection in humans is rare. TTV and TLMV can facilitate active, chronic infections in humans, replicating to produce 3.8 X 10^{10} virions per day [Maggi et al., 2001c]. Infection is characterised by detectable virus both in the bloodstream and in a variety of tissues, which can be the result of either persistent infection with the same genotype or exposure to novel genotypes [Irving et al., 1999; Pollicino et al., 2003]. In spite of the DNA nature of the genome, TTV and TLMV are extremely heterogeneous, with at least 39 genotypes of TTV and 12 TLMV genotypes identified to date.

Although the heterogeneous nature of TTV and TLMV has hampered efforts to characterise these viruses, the discovery of relatively conserved UTR has facilitated the work presented in this thesis. A sensitive and accurate method of differentiating TTV from TLMV was developed using PCR primers that amplify all known genotypes of both viruses. Although only discovered two years after TTV in 1999, relatively little is known about TLMV. At the current time, only 17 paper have been published on this virus, thus the data produced by this study will be invaluable in increasing the understanding of the natural history of TLMV.

An extremely high frequency of Anellovirus infection has been demonstrated in Scottish blood donors, which mirrors the findings of other studies [Biagini and de Micco, 2006; Huang et al., 2001]. The type of Anellovirus was differentiated by analysis of the Tms of the PCR products, revealing approximately one third of samples harboured single TTV infection, one third had TLMV DNA in their serum and one third were infected with TTV and TLMV. The high incidence of dual TTV and TLMV infection has also been described in French blood donors [Biagini and de Micco, 2006] suggesting that individuals can be infected with multiple genotypes of TTV and/or TLMV without any apparent pathology.

SENV D and H, which cluster with TTV genogroup four, are reportedly present at higher titres in the sera of individuals with liver disease than in healthy populations [Sugiura et al., 2004] however, as with TTV, causality has yet to be proven. Although the majority of epidemiological studies have been carried out in Asia [Sugiura et al., 2004], an investigation of the SENV D and H prevalence in the USA found a much lower prevalence of both viruses. Interestingly, the frequency of SENV D and H in Scottish blood donors was the reverse of what has been described in other countries, with a higher proportion of SENV H than SENV D.

The role of the immune system in TTV/TLMV infection has yet to be elucidated. This study has not only shown that the titre of Anelloviruses are higher in the serum of those infected with HIV than patients immunosuppressed following solid organ transplant, individuals infected with HCV or HBV and healthy blood donors, but also that the TTV/TLMV present in the sera of these individuals is more likely to be circulating as free virus than virus complexed with IgG. The titre of TTV and TLMV is also elevated in the transplant recipient groups compared with the other HCV or HBV co-infected and healthy cohorts and again is predominately found as free virus. Although TTV can result in both acute, resolving and chronic infections, chronic infections are more frequent in patients

with immune deficiencies or underlying conditions [Bjoro et al., 2001]. In patients with chronic infections, the presence of circulating quasispecies of TTV has been described along with a high frequency of TTV complexed with IgG [Nishizawa et al., 1999]. It is possible that the sequence divergence described in the hypervariable region of ORF1 resulting in generation of quasispecies facilitates the evasion of TTV from the host immune system, which could explain the high proportion of free virus in HIV positive and transplant recipient samples described in this thesis. It was previously proposed that TTV may establish a commensal relationship with the host [Simmonds et al., 1999] however, the generation of quasispecies suggest selective pressure on the virus to evade the immune system which does not correspond with this hypothesis.

As previously mentioned, attempts have been made to correlate TTV/TLMV infection with disease processes however, a causal role has yet to be established conclusively. Although the ubiquity of the virus described here and in other publications supports the hypothesis that Anelloviruses are not pathogenic, comparison with other small DNA viruses suggests that caution must be exercised. *Parvoviridae* is a family of linear, non-segmented single stranded DNA viruses which infect humans and mammalian species. Parvovirus B19, the only Parvovirus to infect humans, was discovered in 1975 and is relatively common, with an estimated 90% seropositivity in US citizens over 60 years of age. Infection with Parvovirus B19 can result in a number of manifestations that vary in severity depending on the immune status of the infected individual. Immunocompetent individuals can remain asymptomatic or develop erythema infectiosum or slapped cheek syndrome, especially common in children aged six to ten years. It was only in 1981 that the link was made between Parvovirus B19 and severe aplastic anemia in those with underlying conditions or immunosuppression. Human papillomaviruses (HPV) are circular, double stranded DNA viruses which infect epithelial surfaces. Like TTV and TLMV, they are extremely heterogeneous with over 100 genotypes identified. Individuals can be infected with multiple genotypes of these viruses that are not always pathogenic however, certain genotypes are associated with disease processes, namely genotypes 16 are 18, which are known to precipitate cervical cancer. These examples suggest that high prevalence in the general population does not necessarily preclude a pathogenic role for TTV or TLMV. It is possible that certain genotypes of these viruses, possibly in conjunction with immunosuppressed host, can cause disease. Although investigations into this will continue, the nature of the detection methods employed will need to be adapted. The development of the UTR PCR method has contributed to the epidemiological data on

Anelloviruses however, the high prevalence in the general population has largely invalidated any attempt to establish a causal relationship with a disease process. In this study, investigation into elevated TTV/TLMV titres was used to compare populations but the establishment of the genotypes infecting control groups such as non-renumerated blood donor would provide the basis for comparisons with diseased populations.

Elevated titres of TTV/TLMV have determined in the bone marrow and spleen of patients infected with HIV, and more significantly, those that have progressed to the diagnosis of AIDS as defined by the Centres for Disease Control and Prevention [CDC, 1993]. Statistical analysis of the Anellovirus titre showed a significant inverse correlation with $CD4^+$ T cell count although sex and HCV status did not have a significant effect on the titre of TTV/TLMV within this group. Increased TTV/TLMV titres in the sera of HIV infected individuals have been reported previously [Shibayama et al., 2001; Touinssi et al., 2001], as has the presence of TTV in a variety of tissue samples [Okamoto et al., 2001a; Pollicino et al., 2003]. This is the first study to investigate the titre of TTV/TLMV in tissues from HIV positive individuals and AIDS patients. Not only do the data provided here corroborate other evidence of increased TTV/TLMV in people with immunosuppression, limited analysis found paired serum had significantly lower titres of virus than in tissue samples suggesting that both bone marrow and spleen may be sites of virus replication. Both TTV and TLMV are thought to replicate by rolling circle replication and double stranded replicative intermediates have already been discovered in bone marrow [Okamoto et al., 2000d]. In fact, bone marrow has been proposed as the reservoir and primary replication site of TTV following the finding that TTV DNA became undetectable in the myelosuppressed period following bone marrow transplant [Chan et al., 2001].

TTV has been found at increased prevalence in the sera of both transplant recipients [Yokosuka et al., 2000] and in HIV positive patients [Debiaggi et al., 2000] as well as in patients with non A-E hepatitis [Nishizawa et al., 1997], cancer [Nakagawa et al., 1999] and diabetes mellitus [Touinssi et al., 2001]. It may be the case, however, that the increased prevalence is the result of improved detection of the increased titres in those whose immune system is compromised [Touinssi et al., 2001]. This study has validated that theory, conclusively proving that immunosuppression does result in increased TTV/TLMV titres. Unfortunately, it is not possible to draw conclusions about the exact mechanism which results in the elevated Anellovirus titres.

TTV not only infect humans, but also a range of non-human primates. This study further characterised non-primate infection by screening a range of ape and African monkey species for the presence of Anelloviruses and carrying out sequence analysis to compare them to published sequences. A high proportion of all primates species studied were infected with a TTV-like virus, corroborating the high prevalence described by Okamoto and colleagues [Okamoto et al., 2000b]. Alignment of the published sequences as well as those elucidated here showed that the viruses infecting primates were species specific and the genetic diversity of the virus from human TTV and TLMV mirrored the divergence of the host from humans. From this, it was concluded that the viruses evolved along with the host species. Sequence analysis also revealed chimpanzees can be infected with two Anelloviruses which can be classed as TTV-like and TLMV-like. This suggests that TTV and TLMV evolved from a common ancestor and the evolutionary split between TTV and TLMV occurred before the speciation of humans and chimpanzees.

In conclusion, the body of work presented in this thesis has

- shown a high prevalence of TTV/TLMV in Scottish blood donors and for the first time determined SENV D and H prevalence in the same population.
- established for the first time that the immunosuppression associated with AIDS results in an increased TTV/TLMV titre in bone marrow and spleen.
- determined Anellovirus titre is higher in the sera of immunosuppressed HIV positive individuals and transplant recipients than in the HCV or HBV positive sera or in healthy individuals
- confirmed that non-human primates are infected with species-specific TTV/TLMV-like viruses, phylogenetically distinct from human viruses.

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Appendices

9 APPENDICES

Publications arising from this thesis:

- **Thom, K., Morrison, C., Lewis, J.C.M. and Simmonds, P.**

  Distribution of TT virus (TTV), TTV-like minivirus, and related viruses in humans and nonhuman primates.

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Distribution of TT virus (TTV), TTV-like minivirus, and related viruses in humans and nonhuman primates

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Abstract

TT virus (TTV) and TTV-like minivirus (TLMV) are small DNA viruses with single-stranded, closed circular, antisense genomes infecting man. Despite their extreme sequence heterogeneity (>50%), a highly conserved region in the untranslated region (UTR) allows both viruses to be amplified by polymerase chain reaction (PCR). TTV/TLMV infection was detected in 88 of 100 human plasma samples; amplified sequences were differentiated into TTV and TLMV by analysis of melting profiles, showing that both viruses were similarly prevalent. PCR with UTR primers also detected frequent infection with TTV/TLMV-related viruses in a wide range of apes (chimpanzees, gorillas, orangutans, gibbons) and African monkey species (mangabeys, drills, mandrills). These findings support the hypothesis for the co-evolution of TTV-like viruses with their hosts over the period of primate speciation, potentially analogous to the evolution of primate herpesviruses.

Keywords: TT virus; TTV-like minivirus; Circovirus; Untranslated region; Primate; Ape; Old World monkey

Introduction

TT virus (TTV) is a newly discovered nonenveloped virus with a small, covalently closed circular genome of single-stranded DNA. TTV was originally classified as a member of the family *Circoviridae*; this included a number of small DNA viruses with circular, single-stranded genomes that infected several domesticated animal and bird species [chicken anaemia virus (CAV), porcine circovirus (PCV), psittacine beak and feather disease virus (BFDV)]. Alternative proposals for its classification include the designation of TTV as the type member of new virus family, the *Circinoviridae* (Mushahwar et al., 1999). More recently, TTV along with TLMV and CAV have been proposed as members of a new family, the *Paracircoviridae* (Takahashi et al., 2000), as it is now clear that CAV shows greater similarity to TTV and TLMV than it does to other members of the original circovirus family. For example, PCV and BFDV have ambisense genomes, a different virion size and appearance from CAV and TTV, and major differences in genome organisation.

TTV shows considerable genetic diversity, with at least 28 genotypes with nucleotide sequences differing from each other by least 30% (Heller et al., 2001; Okamoto et al., 2001a; Hijikata et al., 1999). These genotypes can be further classified into at least four genetic groups that differ from each other by at least 50% in nucleotide sequence (Hijikata et al., 1999). However, despite this extensive sequence diversity, all variants of TTV share a common genome organisation, with three predicted encoded proteins of similar length and likely function. The great genetic diversity of TTV complicates the interpretation of previous investigations of the prevalence of infection in different human populations, risk groups, and patient cohorts. A polymerase chain reaction (PCR) based method originally used to detect TTV DNA sequences in plasma samples used primers from a region in ORF1 that shows considerable sequence diver-

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sity between TTV genotypes (Okamoto et al., 1998; Simmonds et al., 1998, 1999; Itoh et al., 1999). Detected frequencies of active TTV infection based on this method were therefore invariably underestimates, as TTV variants showing substantial sequence divergence from the prototype strain (TA278) (Nishizawa et al., 1997; Okamoto et al., 1998) were not efficiently amplified (measurements of levels of viraemia in plasma samples were similarly flawed). Combining the results from several subsequent studies that used primers from the more conserved untranslated region (UTR), it appears that TTV infection is universal (or nearly universal) in humans (Okamoto et al., 2001a; Simmonds et al., 1999; Itoh et al., 1999).

Recently, another small DNA virus distantly related to TTV was described (Takahashi et al., 2000). Its name as TTV-like minivirus (TLMV) reflected the fact that its genome was smaller than that of TTV (2915 bases compared with 3852 of the prototype TTV sequence). The difference in size results from a shorter ORF1 encoding the capsid protein and shorter untranslated region. TTV and TLMV show even greater sequence divergence from each other than between genotypes of TTV; however, the precise degree of divergence is difficult to determine as neither the UTR nor the coding sequences from the two viruses can be easily aligned.

Infection with both TTV and TLMV has been detected in a range of nonhuman primates (Verschoor et al., 1999; Abe et al., 2000; Okamoto et al., 2000a,b; Romeo et al., 2000). However, complicating the interpretation of many of the primate studies is the occurrence of cross-species transmission of TTV genotypes. For example, it is known that human TTV variants can infect chimpanzees and macaques (Luo et al., 2000; Tawara et al., 2000). Cross-species transmission may occur in captivity, or through the administration of human plasma-derived blood products containing infectious TTV and TLMV that frequently occur in experimental animals. Beyond primates, the host range of TTV and TLMV is uncertain. One study describes frequent TTV infection of domestic animals, such as cows, pigs, sheep, and chickens. This study used a PCR-based method with primers from the N22 region, implying the presence of genetic variants closely related to the prototype TTV isolate, TA278 (Leary et al., 1999). Whether TTV infection in these species was acquired through contact with humans or vice versa was not determined. More recently, highly divergent TTV-like viruses were detected in pigs, cats, and dogs, distinct from those found using the N22 primers (Okamoto et al., 2002), suggesting that this virus family may indeed be widely or ubiquitously distributed in the mammalian order.

In the current study, we have developed a highly sensitive PCR-based method to detect both TTV and TLMV sequences in plasma and serum using primers from a highly conserved region in the UTR. Detection of amplified DNA sequences in the Roche Lightcycler provided a semiquantitative estimate of the degree of TTV/TLMV viraemia in plasma, while measurement of melting profiles of amplicons allowed TTV sequences to be reliably distinguished from TLMV. The PCR was used to determine frequencies of TTV/TLMV infection in a wide range of wild-caught apes and African monkeys to establish whether infection was indigenous to these species. Finally, using large panels of samples from cows, sheep, goats, and chickens, we determined whether TTV infection was present in domestic animals.

Results

Sequence conservation of TTV and TLMV in the UTR

Despite the extensive sequence divergence of TTV and TLMV in coding regions of the genome, three areas of remarkable conservation have been identified in the part of the UTR that contains transcriptional promoters and splice sites (Kamahora et al., 2000; Okamoto et al., 2000c) (Fig. 1). Sequence conservation is found among all known human isolates of TTV and TLMV, as well as those recovered to date from a range of nonhuman primates. One region is additionally conserved with the very distantly related bird virus, CAV. Based on this sequence alignment, we developed a heminested PCR using primers matching the three conserved regions in the UTR and used them to screen a low-risk, adult human population (blood donors; Table 1). For comparison, each sample was also tested by previously published PCR methods (Okamoto et al., 1998; Leary et al., 1999) using primers from a region of ORF1 corresponding to the originally described N22 clone of TTV (Okamoto et al., 1998) (Table 1). A total of 88 from 100 samples were positive using the UTR primers, but only 5 of 100 were positive using primers from the N22 region (Table 1).

Differentiation of TTV and TLMV sequences

Because of the sequence conservation between TLMV and TTV in the region targeted by the UTR primers, DNA amplified by the UTR primers from the human samples may correspond to either virus or potentially mixtures of both TTV and TLMV genotypes. To investigate the diversity of the detected viruses, we analysed amplified DNA in the Roche Lightcycler. This analysis revealed that melting temperature (T_m) values, as defined by maxima in rate of change of fluorescence with temperature [$d(F1)/dT$], fell into two separate distributions, while some samples contained apparently heterogeneous DNA sequences with maxima in both distributions (examples of melting curves are shown in Fig. 2A). An analysis of T_m values for the 88 PCR-positive blood donors indicated that 34 contained sequences in the high T_m distribution (median value 90.7°C; range 89.0-92.7°C), 18 in the low distribution (median 86.3°C; range 84.5-87.8°C), and 36 with heterogeneous sequences with both T_ms (Fig. 3A).

Sequence composition differences in TTV and TLMV in the amplified region may influence T_m values. TTV se-

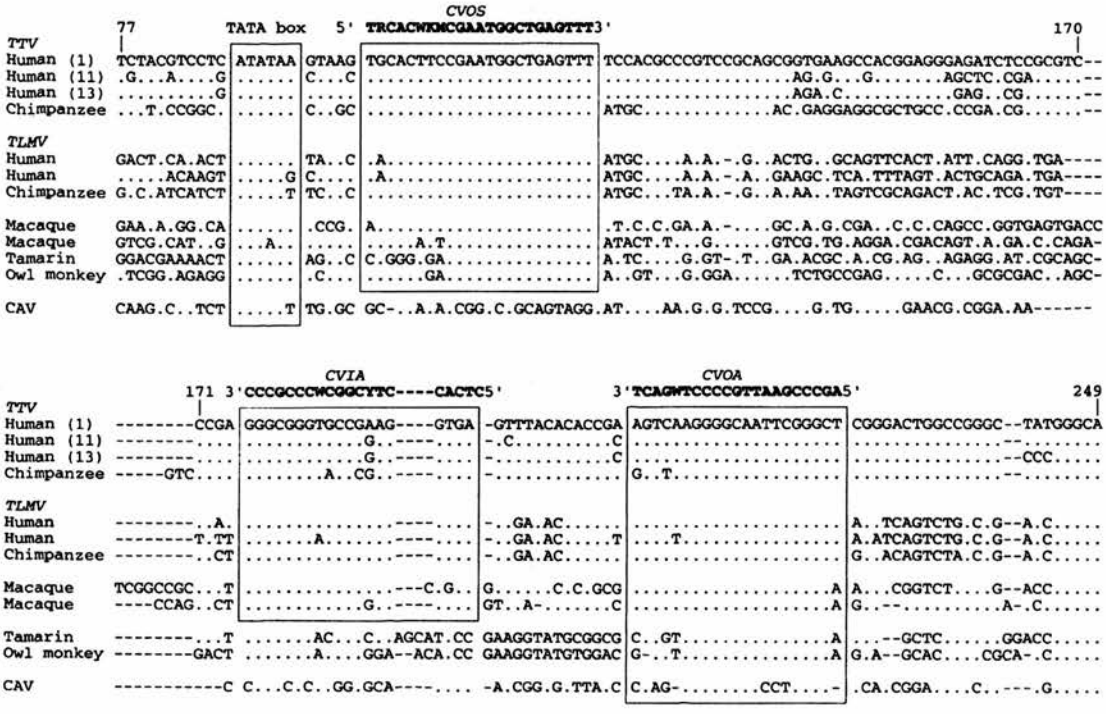

Fig. 1. Alignment of nucleotide sequences from the TTV/TLMV UTR showing the highly conserved region between genotypes 1, 11, and 13 (NC_002076, AB017613, and AB025946), TLMV (NC_002195, AB038629), and homologous sequences of TTV and TLMV from nonhuman primates [chimpanzee (AB041957, AB041963), macaque (AB041958, AB041959), and the new world primates tamarins (AB041960), and owl monkey (AB041961)], and CAV (NC_001427). Conserved regions used to specify primers indicated in boxes. Sequences numbered according to the TTV sequence NC_002195. Symbols: ·, sequence identity with prototype TTV sequence; −, gap introduced to preserve alignment of homologous nucleotide sites.

quences have a higher G+C content than TLMV sequences (mean values of 65.4 and 58.6%, respectively), and TTV sequences are generally 2-4 bases longer. It is therefore possible that the upper distribution of T_m values corresponds to TTV sequences, and the lower distribution of T_m values corresponds to TLMV as both G+C content and amplicon length will increase binding strength. To investigate this, we developed primers that were specific for TTV or TLMV sequences (TTV-OS and TLMV-OS) that bind in the variable region upstream of the TATA box (Fig. 1). A total of 20 samples were amplified using TTV-OS or TLMV-OS with CVOA in the first-round PCR, followed by a nested PCR with CVOS and CVIA. The T_ms of the amplified DNA sequences were compared (Fig. 3B).

Table 1
Detection of TTV and related viruses by PCR

Sample	Number tested	Number positive (%)		
		UTR	N22	N22/2
Apes				
Human	100	88 (88%)	5 (5%)	n.d.
Chimpanzee	5	4 (80%)	0	1 (20%)
Orangutans	14	14 (100%)	0	0
Gibbon	19	12 (63%)	0	0
African monkeys				
Drill	13	12 (92%)	0	0
Mandrill	2	2 (100%)	0	0
Mangabey	4	4 (100%)	0	0
Nonprimates				
Cows	20	0	0	0
Sheep	20	0	0	0
Goats	20	0	0	0
Chickens	29	0	0	0

From the 20 samples, 7 were PCR-positive using the TTV-specific primer, 1 with the TLMV-specific primer, and 6 with both primers (total 14). The T_m values of the amplified DNA showed a strict dependence on the primers used; all 13 samples amplified using the TTV-OS primers showed high T_m values (mean 91°C), while those amplified with TLMV-OS produced DNA with T_m values exclusively in the low distribution (mean 86.8°C). To confirm that the sequences amplified with the virus-specific primers differentiated between TTV and TLMV, amplified DNA from four samples positive with TTV-OS and 4 with TLMV-OS were cloned and sequenced (Fig. 4). In each case, nucleotide sequences corresponding closely to either TTV or TLMV were found in DNA amplified with homologous type primers.

Finally, the cloned sequences were reamplified with CVOS and CVIA, and T_ms determined (Fig. 2B). For each clone, sequences identified by nucleotide sequencing as

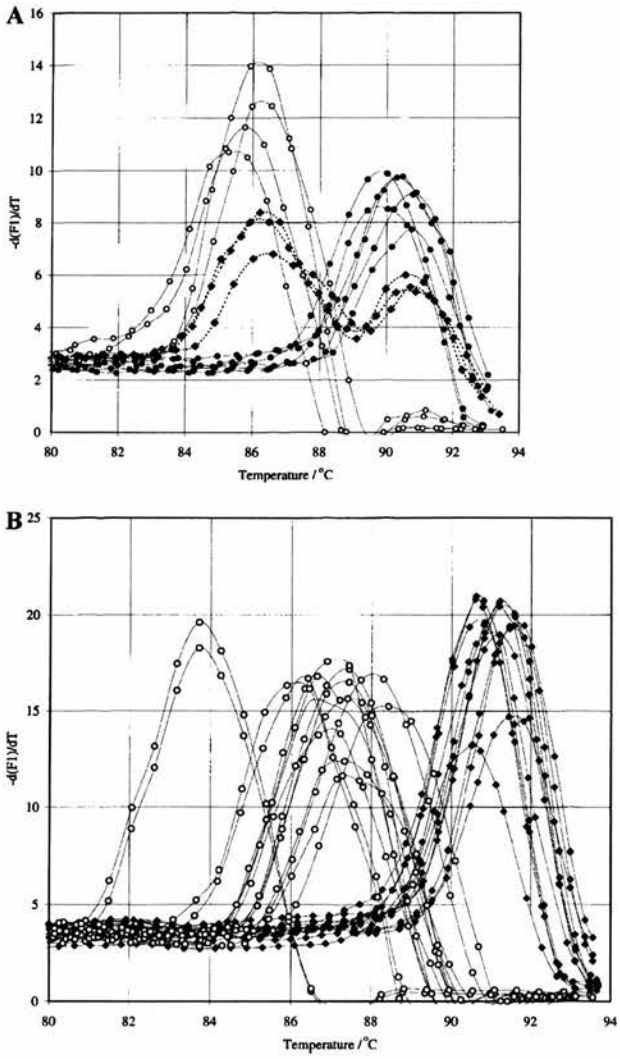

Fig. 2. Melting curve profiles of TTV/TLMV sequences amplified using the UTR primers and analysed in the Roche Lightcycler, plotted as temperature (*x*-axis) vs rate of change in fluorescent intensity (F1) over temperature [*f*(F1)/*dT*]. (A) Amplified DNA sequences from a random selection of TTV/TLMV PCR-positive blood donors. Symbols: ●, samples showing high T_m; ○, low T_m; ◆, evidence for mixed virus population with high and low T_m TTV/TLMV variants. (B) Amplified DNA sequences from molecular clones identified as TTV-like (●) or TLMV-like (○) from sequence comparisons with standard isolates (Fig. 4). The two clones with T_ms of 83.7°C correspond to TLMV1/1 and 1/2 (see text).

being TTV (and originally derived from PCR with the TTV-specific primer) showed high T_m values, while TLMV clones showed T_m values corresponding to the low distribution, as predicted. Interestingly, two sequences (TLMV1/1 and TLMV1/2) that have an additional deletion of five nucleotides in the amplified region (Fig. 4) showed T_m values even lower than observed for other TLMV sequences (83.7°C; Fig. 2B); this observation further indicates the dependence of T_m on sequence length.

Returning to the T_m analysis presented in Fig. 3, it therefore appears that either TTV or TLMV or mixtures of both may predominate in the plasma of individual blood donors.

Analysis of TTV/TLMV variants infecting nonhuman primates

To investigate the frequency of infection of viruses related to TTV and TLMV in other mammals, we screened several species of ape [*Pan troglodytes troglodytes* and *Pan troglodytes vellerosos* (chimpanzees), *Gorilla gorilla gorilla* (western lowland gorilla), *Pongo pygmaeus pygmaeus* (Bornean orangutan) and *Hylobates agilis, Hylobates lar, Hylobates muelleri, Hylobates moloch,* and *Hylobates gabriellae* (gibbons)] and African monkey species [*Mandrillus leucophaeus leucophaeus* (drill), *Mandrillus sphinx* (mandrill), and *Cercocebus torquatus* (cherry-capped mangabey)] by PCR using UTR primers and those based on the N22 gene (Table 1). To investigate the claim that nonprimates may also be TTV positive (Leary et al., 1999), we additionally tested samples from domestic animals (cows, sheep, goats, and chickens) using a PCR protocol and primers identical to those described in the original study (N22/2). High frequencies of viraemia were detected in each of the nonhuman primate species tested using the UTR primers (63–100%), while only one chimpanzee from the 57 nonhuman primates tested was positive using N22-based primers. A total of 89 samples from domestic animals was also tested with each of the three sets of primers. All samples were PCR negative, even using primers and PCR conditions exactly as described previously (Leary et al., 1999).

The distribution of T_m values of DNA amplified from the primate samples differed considerably from those of human samples (Fig. 3C). Amplified sequences from each species showed considerable heterogeneity, and in some cases, heterogeneous sequences with two T_m maxima were observed within a sample (as with TTV/TLMV coinfection in humans). The distribution of T_m values observed in ape samples differed from African monkey species, the former showing a much greater range in values, which, in the case of the chimpanzee samples, corresponded approximately to the range in values observed between human TTV and TLMV variants. To confirm the sequence heterogeneity of the amplified sequences, and to investigate the sequence relatedness of viruses infecting nonhuman primates to human TTV and TLMV, amplified DNA from a selection of chimpanzee, gorilla, orangutan, gibbon, drill, mandrill, and cherry-capped mangabey samples was cloned and sequenced (Fig. 5). The sequences obtained were compared with human TTV and TLMV and previously published sequences from the chimpanzee, two sequences from long-tailed macaques (*Maccaca fasicularis*), two sequences from New World primates [*Aotus trivirgatus* (owl monkey) and *Sanguinis oedipus* (cotton-topped tamarin)], and a sequence from the tree shrew (*Tupaia belangeri chinensis*).

Sequence comparison and phylogenetic analysis was

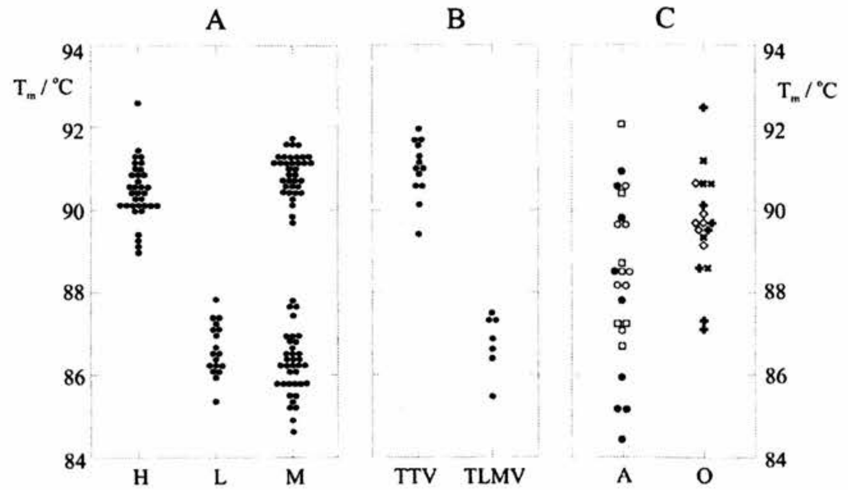

Fig. 3. Distribution of T_m values (corresponding to d(F1)/dT maxima by melting curve analysis; Fig. 2). (A) Samples from the 88 TTV/TLMV-positive blood donors, categorised in high (H), low (L), and mixed (M) distributions. (B) Distribution of T_m values for samples amplified with TTV-specific or TLMV-specific primers (TTV-OS and TLMV-OS). (C) Comparison of T_m values of TTV/TLMV-related viruses directly amplified from serum samples of apes (A) and Old World monkeys (O). Symbols: ●, chimpanzee; ○, orangutan; □, gibbon; ◇, cherry-capped mangabey; ✚, mandrill; ✖, drill.

problematic for this region of genome because of the sequence diversity between variants amplified from different primate species, and between TTV and TLMV, which is so extreme that attempts to align the sequences are necessarily arbitrary and therefore somewhat subjective. For consistency, we have allowed only one gap to be inserted between position 161 and 162 to allow alignment of the downstream primer. This data presentation reveals clearly the length polymorphism of this region, with one particularly long sequence recovered from an orangutan (Pp-E2/2; 18 bases longer than the prototype TTV sequence), and short sequences from the gibbon (Hl-E2) and the tree shrew (Tbc-TTV14; 6–8 bases shorter than the prototype). Because of the alignment problems, we believe that phylogenetic analysis is inappropriate for the UTR sequences, as the method assumes that homologous bases are aligned.

Combined with published sequences, variants infecting chimpanzees fell into two groups, those resembling TTV

	99 CVOS		CVIA 193
NC_002076	TGCACTTCCG AATGGCTGAG TTT	TCCACGC CCGTCCGCAG CGGTGAAGCC ACGGAGGGAG ATCTCCGCGT CCCGA	GGGCG GGTGCCGAAG GTGAG
AB017613	....	....AGA.C... ....GA G..CG.....	....G.. .....
AB025946	....	....AG.G...G ....AGC TC.CGA....	....G.. .....
TTV1/1	.A.....A..	....AGA.CA.. .... TC.-G..... ..-..	....G.. .....
TTV1/2	.TA....A..	....G. ..AGATC..G ....AGA TCG--A.... ..-..	....G.. .....
TTV1/3	.A.....A..	....G. ..AGATC..G ....AGC GA..GA....	....G.. .....
TTV3/1	.A.....A..	....AGA.C... ....CT.....	....G.. .....
TTV3/2	.A.....A..	....AGA.C... ....CT.....	....G.. .....
TTV3/3	.A.....A..	....AGA.C... ....CT.....	....A....... .....
TTV4/1	.A.....A..	....G. ..AGATC..G ....AGC GATCGA....	....A....... .....
TTV4/2	.A.....A..	....G. ..AGATC..G ....AGC GATCGA....	....A....... .....
TTV4/3		A.. ....G. ..AGATC..G ....AGC GATCGA....	....A....... .....
TTV5/1	.A.....A..	....G. ..AGATC..G ....AGC GATCGA..CG T....	....A....... .....
TTV5/2	.A.....A..	....G. ..AGATC..G ....AGC GATCGA..CG T....	....A....... .....
NC_002195	.A........	ATGC... .A.A.G-G.. ACTG..GCAG TTCACT.ATT .CA--G..TG A..A.	....
AB038627	.A........	ATGC... .A.A.G-A.. ACAG..TCA. TTCAGT.ACT CCA--G..TG A..A.	....
TLMV1/1	.A.....A..	ATGC... TA.A.G-G.. GAC....TA. .ATTCA...C TAA------- -.AC.	....A....... .....
TLMV1/2	.A........	ATGC... TA.A.G-G.. GAC....TA. .ATTCA...C TAA------- -.AC.	....
TLMV1/3	.A.....A..	A.GC... TA.A.G-G.. AACGCGTCAG TTACT.ACT. CGG---A.TG ..ATC	....A....... .....
TLMV1/4	.A.....A..	ATGCT.. .A.A.G-A.. GCTGC.CCA. .TCAGT.ACT GCA--G..TG A..A.	....
TLMV2/1	.A........	ATGC... .A.A.G-G.. AC.G..TCA. .ACAGT.ACT CCA--G..TG AA.TT	....A....... .....
TLMV2/2	.A.....A..	A.GC... .A.A.G-G.. GC.G..TCA. TTCAGT.ACT CCA--G..TG A..A.	....A....... .....
TLMV2/3	.A........	ATGC... .A.A.G-G.. AC.G..TCA. .ACAGT.ACT CCA--G..TG AA.TT	....A....... .....
TLMV3/1	.A....GA..	ATGC... .A.A.G-G.. AC.G..GCAG TTTACT.ACT CCA--G..TG A..A.	....A....... .....
TLMV3/2	.A.....A..	ATGC... .A.A.G-G.. AC.G..GCAG TTTACT.ACT CCA--G..TG A..A.	....
TLMV3/3	ATT....A..	A...... .A.A.G-G.. TC.GC.TCA. .AAAGT.C.. GC.--GA.TG AT.TT	....A....... .....
TLMV3/4	.A.....A..	ATGC... .A.A.G-G.. AC.G..TCA. TACAGT.ACT CCA--G..TG AA.TT	....A....... .....
TLMV4/1	.A.....A..	ATGC... .A.A.G-G.. ACAAC.CCAA GTTA.A..CT G.T--G..TG A..A.	....G.. .....
TLMV4/2	.A........	ATGC... .A.A.G-G.. ACAAC.CCAA GTCA.A..CT G.T--G..TG A..A.	....A....... .....
TLMV4/3	.A........	ATGC... .A.A.G-G.. ACAAC.CCAA GTCA.A..CT G.T--G..TG A..A.	....A....... .....
TLMV4/4	.A........	ATGC... .A.A.G-G.. ACAAC.CCAA GTTA.A..CT G.T--G..TG A..A.	....

Fig. 4. Comparison of nucleotide sequences of molecular clones obtained from DNA amplified with TTV-specific (TTV1–TTV5; clone number added as suffix) or TLMV (TLMV1–TLMV4) with previously published sequences of TTV and TLMV (indicated by accession numbers). Sequences numbered according to the TTV sequence NC_002195. Symbols: ·, sequence identity with prototype TTV sequence; −, gap introduced to preserve alignment of homologous nucleotide sites.

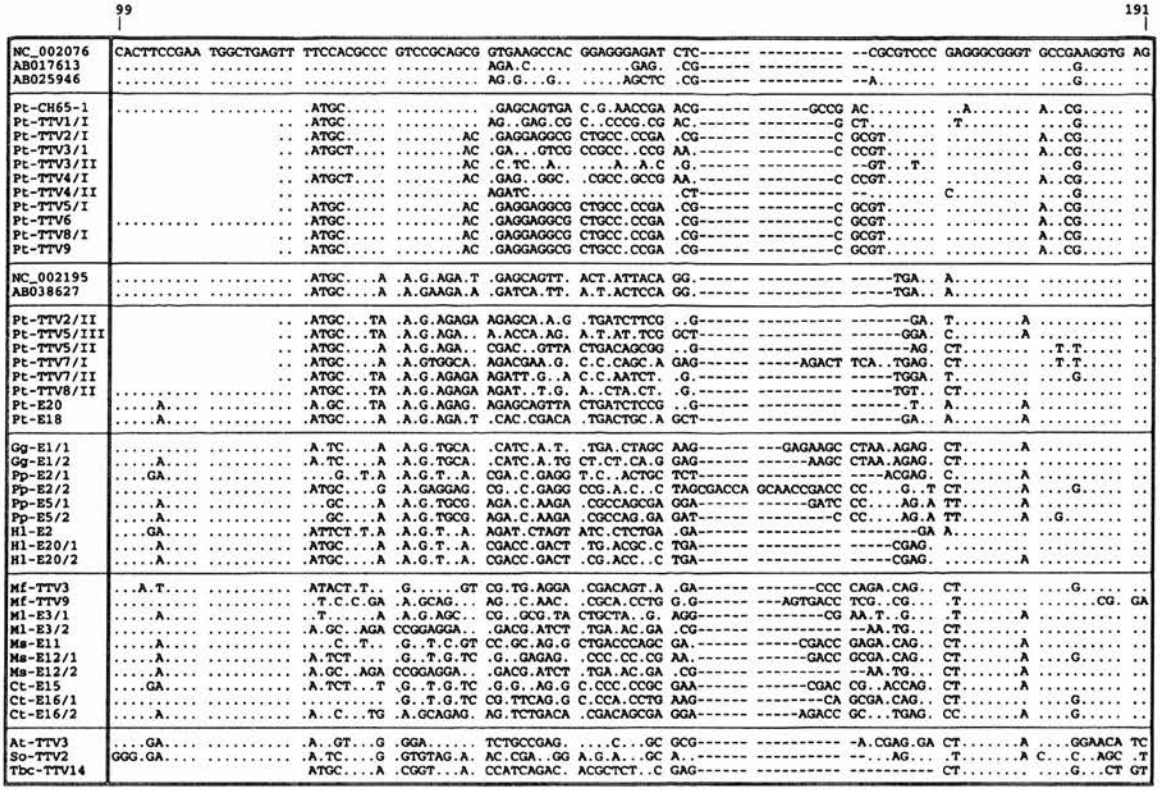

Fig. 5. Comparison of TTV and TLMV UTR sequences with related viruses recovered from primates. TTV sequences: NC_002076 (genotype 1), AB017613 (11), and AB025946 (13); TLMV sequences: NC002195 and AB038627. Origin of nonhuman primate sequences indicated by initials (common name in parentheses): Pt: *Pan troglodytes troglodytes* and *Pan troglodytes vellerosos* (chimpanzee); Gg: *Gorilla gorilla gorilla* (western lowland gorilla); Pg: *Pongo pygmaeus pygmaeus* (Bornean orangutan); Hl: *Hylobates lar, Hylobates agilis, Hylobates muelleri, Hylobates moloch,* and *Hylobates gabriellae* (gibbons); Mf: *Maccaca fasicularis* (long-tailed macaque); Ms: *Mandrillus sphinx* (mandrill); Ml: *Mandrillus leucophaeus leucophaeus* (drill); Ct: *Cercocebus torquatus* (cherry-capped mangabey); At: *Aotus trivirgatus* (owl monkey); So: *Sanguinis oedipus* (tamarin); and Tbc: *Tupaia belangeri chinensis* (tree shrew). New sequences from chimpanzee, gorilla, orangutan, gibbon, drill, mandrill, and cherry-capped mangabey indicated by E prefix; suffixes indicated clone number if more than one sequence obtained from same sample. Sequences were ordered by their genetic relatedness of host species to humans and numbered according to the TTV sequence NC_002195. Symbols: ·, sequence identity with prototype TTV sequence; −, gap introduced to preserve alignment of homologous nucleotide sites.

and those resembling TLMV. Characteristic features that differentiated the sequences included the degree of sequence similarity at the 5′ end of the amplified sequence and overall sequence length; sequences similar to human TLMV also showed 3-6 base deletion compared with the prototype TTV isolate sequence. In contrast, TTV-like sequences from chimpanzees were usually longer than TTV. To demonstrate these sequence relationships graphically, we compared each primate sequence with representative sets of TTV UTR sequences (n = 58, with similar representation of each of the four main genetic groups) and TLMV UTR sequences (n = 50, corresponding to the complete set of nonidentical sequences available on GenBank). Based on the minimal alignment used in Fig. 5, sequences obtained from human subjects (○) fell into two main clusters, one showing 20–30% sequence divergence from TTV but >50% divergence from TLMV sequences, and another more diverse group of TLMV sequences showing 10–35% sequence divergence from TLMV and >50% from TTV (Fig. 6). Interestingly, three sequences of human origin amplified with the TLMV-specific primer showed >50% divergence from all TTV sequence and >45% from TLMV; two of these contained an unusual deletion at position 162 (Fig. 4) and very low T_m values (83.7°C; Fig. 2B), and perhaps correspond to a third (i.e., non-TTV, non-TLMV) genetic group of viruses.

Variants from chimpanzees (●) also fell into two, less well-defined clusters, one corresponding to the TTV-related sequences (n = 10; 24–48%, compared with >50% to TLMV), and the other most similar to TLMV (n = 8, 36–47%, compared to >55% to TTV). In contrast, sequences from other apes, the Old or New World monkey species, and the tree shrew showed no evidence for grouping with human-derived TTV or TLMV sequences, being approximately equally divergent from both (Fig. 6). Even between the limited numbers of sequences obtained from

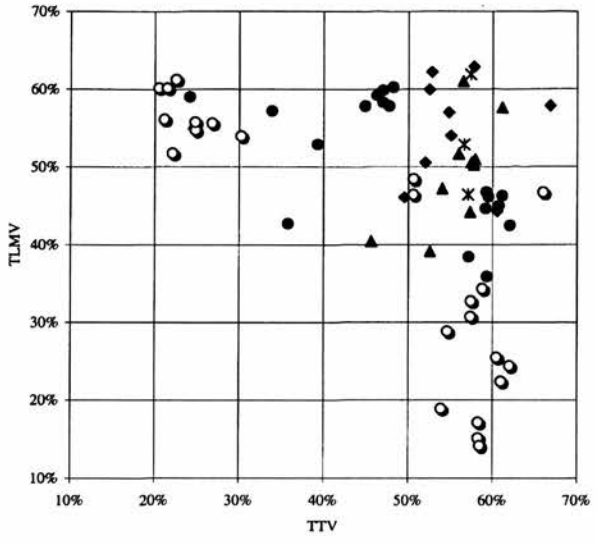

Fig. 6. Sequence divergence of human (this study; Fig. 4) and primate (this study and previously published; Fig. 5) TTV-related UTR sequences from representative sequences of TTV ($n = 58$) and TLMV ($n = 50$) (the full list of reference sequences used is available from the authors on request). Distances from TTV (x-axis) and TLMV (y-axis) sequences shown as uncorrected percentage divergence. Sequence comparisons used the minimal alignment shown in Fig. 5. Symbols were used to indicate different species of origin of UTR sequences; ○, human; ●, chimpanzee; ▲, other apes; ◆, Old World monkey species; *, New World primates and tree shrew.

some species, the genetic diversity within each of the primate species matches the diversity of TTV and TLMV variants found in human samples.

Discussion

Amplification of TTV and TLMV UTR sequences

Despite the great sequence diversity of human TTV and TLMV genotypes, and homologues in nonhuman primates, it is fortunate that conserved regions exist in the UTR to allow a common set of heminested primers to detect a wide range of TTV/TLMV genetic variants. The conserved regions are close to regions involved in transcription, such as the TATA box, and their lack of sequence divergence presumably reflects functional constraints, imposed by their interaction with host replicative machinery (Hijikata et al., 1999). The sequence conservation in this region allowed the development of a heminested PCR capable of amplifying TTV and TLMV viruses in human samples; this method uses primers that match the known variants of TTV and TLMV equally well and is therefore predicted to be equally effective at amplifying each virus from a heterogeneous mixture in a sample. It was therefore possible to carry out a meaningful comparison of their relative frequencies and abundance in human samples.

Among the 88 PCR-positive samples using the UTR primers, approximately one-third contained exclusively TTV sequences, one-third contained TLMV, and one-third contained mixed infection of TTV and TLMV based upon the T_ms of the amplified DNA measured on the Roche Lightcycler. These results do not rule out the absence of infection with both viruses in any of the samples, as amplified sequences with a low relative abundance may not be detectable by the melting curve analysis. To determine the frequency of infection with each, virus-specific primers would be required. However, our findings indicate that TLMV infection is equally prevalent to TTV in humans and that both viruses can coexist and replicate to similar titres in a substantial proportion of individuals. These findings are consistent with previous observations of the presence of multiple TTV genotypes in human plasma and tissues (Ball et al., 1999; Okamoto et al., 1999; Forns et al., 1999; Niel et al., 2000). The finding that plasma itself is not representative of the range of greater genetic variants that may be sequestered in tissues such as bone marrow, lymphoid tissue, and liver (Okamoto et al., 2001a) suggests that infection with both TTV and TLMV may be extremely widely distributed or possibly universal in the human population.

Detection of TTV/TLMV homologues in nonhuman primates

The sequence conservation of the UTR primers allowed an investigation of the distribution of TTV/TLMV-related viruses in a wider range of nonhuman primates than previously attempted. The primer sequences matched previously published sequences from chimpanzees and the macaque (an Old World monkey) (Fig. 1) (Inami et al., 2000; Okamoto et al., 2000b). There was also reasonable sequence conservation with TTV/TLMV sequences recovered from New World primates and the tree shrew (Okamoto et al., 2001b), although the sequence differences with the inner antisense primer would undoubtedly compromise the efficiency of the UTR PCR for these more divergent viruses.

Despite the sequence diversity of primate viruses and the potential problem this poses for PCR-based screening, close to universal infection was found in each ape and Old World monkey species screened (Table 1). Comparisons of amplified sequences from the UTR of each species confirmed it was improbable the viruses were human in origin; none corresponded to known human genotypes of TTV or TLMV (Fig. 5 and data not shown). Furthermore, amplified DNA from each of the primate species (particularly the African species) showed a distribution of T_ms quite distinct from the narrow ranges found among the TTV and TLMV sequences amplified from human subjects (Fig. 3C).

The great sequence diversity of the UTR sequences prevented any defensible strategy for the alignment of homologous bases. On this basis, it was felt unjustified to attempt to carry out formal phylogenetic analysis. However, inspection of the minimal alignment (Fig. 5) and sequence diver-

gence from representative data sets of human-derived TTV and TLMV sequences (Fig. 6) indicated that the division of the chimpanzee UTR sequences into TTV and TLMV groups was potentially justifiable, principally on the basis of the sequence at the 5′ end of the amplicon (positions 115-122), although the "ATGC" motif found in human TLMV sequences at position 120 is also present in the TTV-like chimpanzee sequences. The assignment of chimpanzee sequences into TTV- and TLMV-related groups is also supported by their complete genome sequences; apart from their greater sequence similarities, Pt-TTV6 has a genome length of 3690 bases, comparable to that of the prototype human TTV sequence, NC_002076 (3853 bases), while Pt-TTV8/II is only 2785 bases, comparable to the TLMV variant NC_002195 (2860 bases) (Okamoto et al., 1998, 2000b). This finding suggests that the evolutionary split between TTV and TLMV therefore must have occurred before the speciation of humans and chimpanzees (7 Myr) if TTV/TLMV-related viruses coevolved with their hosts (see below).

In contrast to the chimpanzee-derived UTR sequences, sequences from the other apes and African monkey species analysed in this study showed no specific relationship to TTV or TLMV (Fig. 5), being equally dissimilar to human-derived TTV and TLMV sequences (Fig. 6). Supporting this conclusion, the complete genome sequences from macaques (Mf-TTV3 and -TTV9, also dissimilar to TTV or TLMV in the UTR; Fig. 5) are 3798 and 3763 bases in length, but show no close sequence relationship to either human or chimpanzee TTV sequences (Okamoto et al., 2000b).

Despite the difficulties associated with the comparison of highly divergent sequences in the UTR, and elsewhere in the genome (Okamoto et al., 2000b), none of the data obtained in the current study is inconsistent with the hypothesis of coevolution of TTV/TLMV-related viruses with primates over several tens of millions of years. Evidence for cospeciation includes the increasing divergence of Old and New World primate variants to human TTV/TLMV (Figs. 5 and 6) (Okamoto et al., 2000b), and the outlier phylogenetic position of the TTV/TLMV-related virus recovered from tree shrews (Okamoto et al., 2001b), reflecting the depth of the split of the host species from other species in this primate order. In many respects, this scheme corresponds closely to the proposed coevolution of herpesviruses in primates and other mammals (McGeoch et al., 2000). While the three groups of herpesviruses (α, β, and γ) are represented in each mammalian order investigated and presumably diverged before or during their original differentiation 90 million years ago, some γ-herpesviruses show evidence for more recent evolutionary splits and cocarriage during the period of primate evolution, analogous to the TTV/TLMV split suggested by the comparison of sequences from some ape species.

Detection of TTV/TLMV-related viruses in other mammals

The previous finding (Leary et al., 1999) of TTV infection in several domestic animals (pigs, cows, sheep, and chickens) is clearly inconsistent with the hypothesis of TTV/TLMV coevolution in primates, particularly as the isolates from farm animals were not apparently genetically distinct from those found in humans (Leary et al., 1999). To resolve this discrepancy, we attempted to reproduce the findings of the study using a nationally collected set of serum samples from cows, sheep, goats, and chickens. While this was by no means a comprehensive selection of farm animals, the high frequencies of TTV detection reported previously in a probably similarly geographically restricted sample collection suggested that the materials available for this study would suffice to reinvestigate the issue.

To detect TTV infection, we used PCR methods involving UTR primers (this study), N22 primers used extensively in previous surveys of human and primate TTV prevalences (Okamoto et al., 1998), and a PCR method that precisely reproduced the experimental conditions of the original study (Leary et al., 1999). All 89 samples tested were negative with each of set of primers and PCR conditions (Table 1). While we cannot rule out that domestic animals may vary in TTV prevalence between countries, another interpretation is that the previously reported findings are incorrect. Returning to the coevolution hypothesis, the increasing sequence divergence of TTV/TLMV-related viruses from human variants observed in primate screening, including the appearance of sequence differences in the primer sites (Fig. 1), suggests that TTV/TLMV-related viruses split from primate variants even earlier in mammalian evolution (90 Myr) and may therefore be refractory to amplification with even with heminested UTR primers. Very recently, evidence for the existence of the predicted highly divergent viruses in nonprimates has indeed been obtained using nonnested primers for the UTR corresponding approximately in position to CVOS and CVOA (Okamoto et al., 2002). Complete genome sequences from cat-, dog-, and pig-derived TTV-related viruses were obtained by reverse PCR from the amplified UTR region and found to be even more divergent from the prototype human TTV than those from any of the primates or the tree shrew. None of the nonprimate viruses showed any significant sequence similarity to the N22 primers.

In summary, the use of direct nucleotide sequencing combined with melting curve analysis of amplified DNA sequences documents the extreme sequence diversity of TTV, TLMV, and related viruses in humans and nonhuman primates. With the limitations on primer design that results from this genetic variability, we have also shown that infection with this virus family is likely to be ubiquitous or near ubiquitous in primates at least down to the Old World monkey/ape split. In terms of their ubiquity and persistence, TTV and TLMV are comparable to the distributions of

herpesviruses, but differ uniquely in their high-replicative capacity that leads to life-long persistent viraemia in most individuals. Their genetic diversity greatly exceeds that of any other known virus, even that of the papillomaviruses infecting the skin. Investigating the mechanisms by which TTV, TLMV, and related viruses can establish multiple, largely, or entirely nonpathogenic persistent infections in primates is clearly a fertile area for fundamental virology and immunology research in the future.

Materials and methods

Samples

A total of 100 human plasma samples from Scottish blood donors were available for investigation of TTV/TLMV prevalence in a human population. All samples were negative for antibodies to human immunodeficiency virus type 1 and 2 and hepatitis C virus, and negative for hepatitis B virus surface antigen. Sera from 13 drills, 2 mandrills, 4 cherry-capped mangabeys, 5 chimpanzees, 14 orangutans, 19 gibbons, and 1 gorilla were available for investigation of TTV/TLMV infection in nonhuman primates. Sanctuaries in Nigeria, Cameroon, and Taiwan supplied these samples, with the exception of the gorilla sample supplied by Edinburgh Zoo, and all the primates used had originally been wild-caught. Each primate species was housed separately from other primates. Finally, we tested sera from 20 cows, 20 sheep, 20 goats, and 29 chickens collected from a variety of locations in England and Scotland.

Extraction of nucleic acid

Nucleic acid from 2 ml human plasma was extracted using a guanidinium thiocyanate/silica-based method (Nuclisens, Organon-Teknika, Boxtel, Netherlands) and eluted in 50 μl Tris–HCl buffer. Nucleic acid from all other samples was extracted from 100 μl serum using a standard phenol–chloroform protocol and precipitated with ethanol. The resulting pellet was resuspended in 25 μl nuclease-free water.

Amplification of TTV and TLMV

Three sets of primers were utilised during this study to allow a comparison of primer sensitivity and of virus prevalence in different host species. Heminested primers NG059, NG061, and NG063 (primer set N22), derived from the original N22 clone, have been used extensively in previous investigations of TTV epidemiology (Okamoto et al., 1998). Fully nested primers (primer set N22/2), originally published in conjunction with a study of TTV viraemia in farm animals, were also investigated (Leary et al., 1999). Conditions for PCR using these primers were as previously described (Okamoto et al., 1998; Leary et al., 1999). The third primer set (primer set UTR) is heminested and spans a conserved region of the UTR. Ambiguities were included in the primer design to allow TLMV to be coamplified. In the first round of the PCR 5 μl of extracted DNA was amplified using primers CVOA and CVOS (sequences 5′-AGCCCGAATTGCCCCTWGACT-3′ and 5′-TRCACWKMCGAATGGCTGAGTTT-3′). Two microliters of this reaction was carried over to a second round and amplified using primers CVOS and CVIA (sequence 5′-CTCACCTYCGGCWCCCGCCC-3′), resulting in a 95-bp amplicon. Amplification conditions were 94°C/18 s, 55°C/21 s, and 72°C/90 s for 30 cycles, with an additional 6 min extension at 72°C to finish. PCR products were visualised on a 2% agarose gel stained with ethidium bromide.

Differentiation of TTV and TLMV using real-time PCR

The first-round PCR using UTR primers was carried out as above, and 2 μl of the reaction product was used in a second round which was performed on a Lightcycler (Roche Diagnostics GmbH) using primers CVOS and CVIA and Lightcycler-DNA Master SYBR Green I (Roche Diagnostics GmbH). Amplification of the template (initial denaturation 95°C/30 s, then 30 cycles of 95°C/0 s, 55°C/5 s, and 72°C/10 s) was followed by melting curve analysis, during which the samples are heated from 60 to 95°C to allow the determination of the melting temperature (T_m) of the amplified DNA sequences. The analysis of this melting curve produced a peak with a virus-specific T_m, which we used to differentiate and characterise the products.

Molecular cloning and sequencing of TTV and TLMV DNA

Human samples positive with the UTR primers were subjected to a second nested PCR in which primer CVOS in the first round was replaced with a TTV- or TLMV-specific primer [TLMV specific primer 5′-GGAGGAGHHWMHMHACTATATAA-3′ (TLMV-OS) and TTV specific primer 5′-AACMKGGTCTACRTCCTSATATAA-3′ (TTV-OS)]. The second round of the PCR and conditions are as described for UTR primers, and products were run on the Lightcycler to ensure the correct virus had been amplified. Primate samples were amplified with the UTR primers prior to cloning. PCR products were ligated into pGEM-T Easy Vector (Promega Corp., Madison, WI) and cloned according to manufacturer's instructions. Nucleic acid was purified using QIAprep Spin Miniprep kit (Qiagen).

Nucleotide sequencing and sequence analysis

Miniprepped DNA was sequenced from both ends by manual cycle sequencing using a thermostable DNA polymerase (Promega) and primers specific to the M13 portion of the vector. Sequences were analysed using Simmonic 2000 sequence analysis package. Sequences obtained in this

study have been submitted to GenBank and have been the assigned the Accession Nos. AY187821–AY187866.

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PROGRESSION TOWARDS AIDS LEADS TO INCREASED TORQUE TENO VIRUS AND TORQUE TENO MINIVIRUS TITRES IN TISSUES OF HIV INFECTED INDIVIDUALS.

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Abstract

Torque Teno virus (TTV) and Torque Teno minivirus (TTMV) are highly prevalent in the general population and although no disease has been associated with these viruses yet, co-infections with other pathological viruses are frequent. Both viruses are extremely heterogeneous, especially for DNA viruses, and the role of the immune system in controlling the infections has yet to be established. In this study the TTV/TTMV viral loads in HIV positive tissues have been investigated for the first time. The titres of both TTV and TTMV were compared in the bone marrow and spleen tissues from 3 groups: HIV negative individuals, HIV positive individuals and HIV positive individuals who had progressed to AIDS, leading to immunosuppression. Limiting dilution PCR using primers situated in the UTR region of the genome were used to semi-quantitate the virus, and TTV and TTMV were differentiated using melting curve analysis of the PCR product. The AIDS group had significantly higher titres compared with both the HIV positive and negative groups for both bone marrow (AIDS vs. HIV positive $p=0.006$, AIDS vs. HIV negative $p<0.001$) and spleen (AIDS vs. HIV positive $p=0.022$, AIDS vs. HIV negative $p<0.001$). Analysis of TTV/TTMV titre with CD4 T lymphocyte count showed a significant inverse correlation however neither HCV co-infection or type of Anellovirus infection (single TTV or TTMV, or mixed TTV/TTMV) showed any significant correlation with virus titre. The results show a link between deterioration of the immune system and increased viral loads in studied tissues.

Introduction

TTV was originally isolated from the serum of an individual (T.T.) with post-transfusion non-A-E hepatitis [Nishizawa et al., 1997] in 1997. Further analysis revealed it to be a small, circular, unenveloped, single stranded DNA virus of 3.8kb, the first virus of this type known to infect humans. TTMV was discovered in 1999 when primers designed to amplify TTV were found to be partially homologous to a region of TTMV [Takahashi et al., 2000]. Although they share similar genomic organisation, the TTMV genome is approximately 1kb smaller than that of TTV. Recently the decision was made to name TTV as the type virus of a newly created genus Anellovirus which will also contains TTMV and the newly described Small Anellovirus (SAV) [Jones, 2005].

Over 90% of adults are infected with TTV and/or TTMV and it is not uncommon for individuals to be infected with multiple genotypes of one or both of these viruses [Okamoto et al., 2001; Saback et al., 2002; Thom et al., 2003]. The route of transmission that results in such a high prevalence in the general population is unclear, however TTV DNA has been identified in peripheral blood, fecaes, saliva and semen, indicating the possibility of parenteral, fecal oral and sexual transmission [Matsubara et al., 2000; Nishizawa et al., 1997]. TTV has been detected in a number of different tissues and cell types including liver, thyroid gland, lymph node, spleen and pancreas [Okamoto et al., 2001], and has also been shown to be present in high titres in bone marrow cells. The method or site of replication is not fully understood at this point in time, however there is a body of evidence to suggest that TTV might replicate in bone marrow. Double stranded replicative intermediate forms of TTV DNA and 3 mRNA species have

been isolated from the bone marrow of TTV positive individuals [Okamoto et al., 2000a; Okamoto et al., 2000b] and TTV levels in the serum of TTV positive patients became undetectable during the period of myelosuppression following bone marrow transplant [Kanda et al., 1999] suggesting a role for hematopoietic cells in maintaining the viraemic status of TTV positive individuals.

No pathological role for TTV/TTMV infection has been established, however the high prevalence of virus in the general population leads to frequent co-infections with other viruses. The immune dysfunction associated with human immunodeficiency virus (HIV) can result in opportunistic infections and the reactivation of latent viruses, so the natural history of TTV/TTMV in HIV positive individuals is of particular interest.

The role of TTV/TTMV in HIV infection and the progression towards AIDS is still uncertain with many studies describing conflicting results on the significance of co-infection. A Danish HIV cohort had significantly higher prevalence and titre of TTV compared with blood donors (76% vs 7%) [Christensen et al., 2000] however the number of blood donors testing positive for TTV appears to be particularly low. This is probably due to the use of PCR primers sited in ORF2 of the TTV genome which would restrict the genotypes of virus amplified. Further analysis of HIV positive cohorts showed a significant inverse correlation between TTV titre and CD4 T^+ cells [Christensen et al., 2000; Shibayama et al., 2001] and high serum TTV concentration was also cited as an independent factor associated with decreased patient survival [Christensen et al., 2000]. These studies appear to indicate a role for the immune system in the control of TTV/TTMV replication, however a study following changes in TTV

and TTMV viral load in individual cases failed to find a significant increase in titre following progression to AIDS or in conjunction with changes in CD4 and CD8 T lymphocytes counts [Moen et al., 2002].

The high number of co-infections of HIV and hepatitis C virus (HCV) and the high prevalence of TTV in HIV positive populations invariably also result in HIV, HCV and TTV co-infections. Although it is now thought to be unlikely that TTV is the cause of non A-E hepatitis, there is evidence that infection with TTV combined with dual HIV and HCV infection increases the alanine amino transferase (ALT) levels in patients indicating more severe hepatitis than in patients infected only with HIV and HCV [Shieh et al., 2003]. It has even been suggested that patients infected with TTV and HIV have lower ALT levels than those infected with HIV alone [Sherman et al., 2001].

In this study, the titres of TTV and TTMV in bone marrow and spleen taken from HIV positive and negative individuals and have been investigated and, in the case of the HIV positive cases, an attempt has been made to correlate the titre of Anellovirus with the CD4 count. This is thought to be the first investigation into the titre of TTV/TTMV in HIV positive tissues.

Materials and methods

Patient samples

Tissue samples of bone marrow and spleen were obtained at autopsy and held in the Brain and Tissue bank of Edinburgh and the MRC Tissue Bank for the Investigation of Sudden Death (Western General Hospital, Edinburgh, UK). Nineteen tissue samples were from a cohort of HIV infected individuals and of those in 13 (12 male, 1 female, median age 32 years) the cause of death was AIDS related while the cause of death in the remaining 6 (3 male, 3 female, median age 29 years) was unrelated to their HIV status. A further 7 (4 male, 3 female, median age 70 years) HIV negative tissues were also used in this study. Patient information is summarised in Table I. Where available, CD4 T cell counts were obtained from medical records. All of the samples were stored at -40°C prior to DNA extraction. The use of tissue samples was approved for research by the Lothian Ethics of Research committee.

DNA extraction and TTV amplification

Nucleic acids were extracted from a 25-50mg section of frozen bone marrow and spleen tissue using a High Pure PCR Template Preparation Kit (Roche Applied Sciences, Mannheim, Germany), according to the manufacturers instructions for isolation of nucleic acids from mammalian tissue. NA was eluted into 200µl of elution buffer. Total DNA concentration was estimated by spectrophotometry at 260nm.

The oligonucleotide primers used were situated in the partially conserved untranslated region of the genome ambiguities were included in the primer sequences to allow both TTV and TTMV to be amplified simultaneously. In the primary PCR reaction, a volume of DNA equivalent to 0.1μl was added to a total volume of 50μl containing 5μl 10X PCR buffer, 30μM of dATP, dTTP, dCTP and dGTP, 1 unit *Taq* DNA polymerase (Promega UK, Southampton, UK) and 0.25μM of primers CVOS and CVOA [Thom et al., 2003]. A secondary reaction was carried out by transferring 2μl of the primary reaction to a final reaction volume of 20μl in which the primers CVOS and CVIA were used. Both the primary and secondary PCRs were amplified using 30 cycles of 94°C for 18 seconds, 55°C for 21 seconds and 72°C for 90 seconds. PCR products were visualised using gel electrophoresis on a 2% agarose gel stained with ethidium bromide. Semi-quantitation of TTV/TTMV was carried out by amplification of serial 10-fold dilutions of DNA where the last PCR positive dilution was used to estimate the titre of virus in the tissue. Titres were expressed in terms of number of copies per million cells, estimated using the assumption that a human diploid cell contains 6.6pg of DNA.

HCV amplification

Hepatitis C amplification was carried out using nested primers Z4130 and Z4133 in the primary PCR and Z4132 and Z4132 in the secondary PCR as described by Cleland and colleagues [Cleland, 1999].

Real Time PCR

To differentiate between TTV and TTMV, 2μl of primary PCR product was subjected to PCR amplification and melting curve analysis on a Roche lightcycler as previously described [Thom et al., 2003].

Statistical analysis

Statistical analysis was carried out using SPSS (version 11 for Macintosh, London, UK). Comparison of unpaired observations was analysed using the Mann-Whitney U test. The Wilcoxon signed tanks test was used to compare paired data and the Spearmans rank order correlation to test for associations between variables. For all methods a p-value > 0.05 was considered not significant.

Results

Titre of TTV/TTMV in bone marrow and spleen tissues.

Samples from three groups of individuals were investigated in this study: HIV positive individuals who died of AIDS defining diseases [CDC, 1993], pre-AIDS individuals whose cause of death was unrelated to their HIV status, and HIV negative individuals. Bone marrow and spleen tissue was obtained during autopsy from a total of 13 AIDS, 6 pre-AIDS (HIV positive) and 7 HIV negative patients (Table I). All of the tissues were positive for TTV/TTMV when amplified using the UTR primers therefore limiting dilution PCR was used to semi-quantitate the titre of virus in each of the tissues. The results of this are summarised in Table II and Table III. Four of 13 (30.7%) pairs of samples from the AIDS cohort, 2/6 (33.3%) from the pre-AIDS groups and none of the HIV negative individuals were positive for HCV.

The median virus titre was similar for bone marrow and spleen in each of the three groups: AIDS group had a median titre of $7x10^7$ copies per million cells in both bone marrow and spleen (range; bone marrow: $7x10^6$ to $7x10^8$ copies, spleen: $7x10^4$ to $7x10^8$ copies), the median titre was $3.6x10^5$ for HIV positive bone marrow (range $7x10^2$ to $7x10^7$) and $7x10^4$ for HIV positive spleen (range $7x10^2$ to $7x10^8$). The lowest titres were seen in the HIV negative group (bone marrow: median $7x10^4$, range $7x10^1$ to $7x10^4$, spleen: median $7x10^3$, range $7x10^2$ to $7x10^4$). Titre was also similar between the two tissues from the same individual, with 71-100% of all samples having titres of virus in spleen within two logs of the bone marrow titre (Figure I). Differences between the titre of TTV/TTMV in bone marrow and

spleen for individuals within the 3 groups were not found to be significant (AIDS $p = 0.24$, HIV positive $p = 0.68$, HIV negative $p = 0.59$).

Analysis to compare the titre of TTV/TTMV in bone marrow between the three groups showed significantly higher titre of virus in the AIDS cohort compared with both the HIV positive group ($p = 0.006$) and the HIV negative group ($p < 0.001$). Significantly higher titres were also seen in the AIDS cohort when the TTV/TTMV was quantified in spleen (AIDS vs HIV positive: $p = 0.022$, AIDS vs HIV negative: $p < 0.001$). This supports the role of the immune system in regulating TTV/TTMV titre although the exact mechanism of this remains unclear. To further investigate this, $CD4^+$ T cell counts were obtained for 9 of the 13 members of the AIDS cohorts and for 5/6 HIV positive individuals. There was a significant inverse correlation between $CD4^+$ T cell count and TTV/TTMV titres in both bone marrow ($p = 0.007$) and spleen ($p = 0.008$) indicating that either the depletion of this cell subset or the changes in the immune system associated with the progression towards AIDS may facilitate either the increased replication of these viruses or a reduced rate of viral clearance.

Differentiation of TTV and TTMV from bone marrow and spleen using real time PCR

The UTR primers utilised in the study are able to simultaneously amplify TTV and TTMV enabling an insight into the prevalence of Anelloviruses in general within bone marrow and spleen. Given the significantly increased viral load found in autopsy tissues isolated from HIV positive individuals who had progressed to AIDS, it was important to differentiated between the two viruses to assess if the increased viral load was predominately due to TTV or TTMV. A

previously reported a method of employing melting curve analysis was utilized to distinguish between TTV and TTMV [Thom et al., 2003] in the bone marrow and spleen tissue samples (Figure II and Figure III). Interestingly, the highest percentage of mixed TTV and TTMV infections occurred in HIV negative bone marrow and spleen. Mixed infection was not significantly associated with TTV/TTMV titre, $CD4^+$ T cell count, age, or sex ($p > 0.05$).

Of the 26 pairs of bone marrow and spleen samples investigated, 19 (73.1%) had the same type of infection in both tissues (i.e. single TTV infection, single TTMV infection or mixed TTV and TTMV infection). Four of the seven pairs which had different infections in the 2 tissues formed part of the AIDS cohort and 3 were from HIV negative individuals. All showed mixed infection in one of the tissues and single TTV or TTMV infection in the corresponding second tissue apart from 1 individual, a 28 year old female who died of AIDS related diseases. Melting curve analysis showed TTV infection of the bone marrow and TTMV in the spleen of this individual.

Impact of HCV status in HIV positive and negative tissue samples

Due to the high frequency of HIV/HCV co-infections, it was considered appropriate to firstly determine the HCV status of the patients whose tissues were used in this study and secondly to determine if HCV status impacted on the titre of TTV and TTMV in tissues.

Of the 26 patients tested, 6 were infected with HCV, including 4/13 (30.8%) of the AIDS cohort, 2/6 (33.3%) of the HIV positive cohort and 0/7 HIV negative individuals. The difference in TTV/TTMV titres between samples co-infected

with HCV and HIV compared with those infected with HIV alone was investigated. The p-value was 0.69 for bone marrow and 0.52 for spleen indicating that co-infections of HCV and HIV did not significantly alter the titre of TTV/TTMV in the bone marrow and spleen samples.

Discussion

The compromised immune system resulting from HIV infection is well documented, as is the risk of opportunistic infections as immunosuppression becomes more marked on progression towards AIDS. Co-infections with other viruses are common in HIV infected patients and evidence is mounting that these can result in the modification of the HIV disease processes, for example it is thought the Flavivirus GBV-C has a positive impact on disease progression in HIV infected patients [Northfield, 2005].

TTV and TTMV are small, circular, DNA viruses which can be transmitted through parenteral, non-parenteral and faecal oral routes [Puig-Basagoiti et al., 2000; Tawara et al., 2000]. Since they share some of the same transmission routes as HIV, TTV and/or TTMV co-infections with HIV are common. TTV was originally thought to be a candidate novel hepatitis virus and although its role in liver disease remains controversial, until its pathogenic potential is fully characterised it will remain relevant to investigate the implications in those with immune dysfunctions.

In this study, semi-quantitation of the Anellovirus titre in bone marrow and spleen tissues from three cohorts: HIV positive individuals who died of AIDS related infections, HIV positive patients who died pre-AIDS and HIV negative individuals was carried out. Significantly increased viral load in tissues removed from those with an AIDS defining illness compared with both the HIV positive and negative groups were found, suggesting that the immune system does have some role of regulating TTV and TTMV viraemia. A significant

association between CD4+ T cell count TTV/TTMV titre was also established, corroborating studies showing an inverse relationship between TTV viral load and level of immunosuppression [Shibayama et al., 2001; Touinssi et al., 2001]. It has been suggested that 90% of TTV particles are cleared from plasma every day [Maggi et al., 2001] so the increased titre reported here could be indicative of a reduction in viral clearance however, to conclude that CD4+ T cells are in some way central to the clearance of TTV is contrary to studies suggesting that the immune reconstitution associated with high-activity antiretroviral therapy (HAART) did not lead to a decrease in TTV viraemia [Takamatsu et al., 2001]. The role of the immune system in the natural history of these viruses is further implicated by the evidence that TTV DNA became undetectable in patients during the myelosuppressed period following bone marrow transplant [Kanda et al., 1999], possibly due to the depletion of hemic cells which act as a reservoir for TTV. There was no correlation between the TTV/TTMV viral load in bone marrow and spleen and HCV status, age or sex ($p > 0.05$).

It is hypothesised that TTV maybe able to replicate in the haematological compartment and double stranded replicative intermediates have been found in the bone marrow as well as in liver in humans [Okamoto et al., 2000b; Okamoto et al., 2000c] and in the liver, bone marrow and small intestine of experimentally infected rhesus monkeys [Xiao et al., 2002]. Peripheral blood mononuclear cells (PBMCs) were thought to act as a reservoir for TTV but double stranded intermediates have not been isolated from these cells suggesting that this is not a site of replication [Okamoto et al., 2000b]. However, TTV replication has been achieved in vitro by stimulation of PBMCs with phytohemagglutinin, lipopolysaccharide, and interleukin-2 [Desai, 2005; Mariscal et al., 2002]. Similar

titres of TTV/TTMV in bone marrow and spleen suggests either the spleen is also a site of replication or the infiltration of spleen by cell types supporting replication.

The use of PCR primers, which can simultaneously amplify both TTV and TTMV, and the real time PCR method of differentiating between them provides much needed data on the relationship between these viruses. These data have shown that like TTV, TTMV can infect and possibly replicate in bone marrow and spleen tissue both independently and in the presence of TTV. Surprisingly, the highest percentage of dual TTV and TTMV infection occurred in the bone marrow of the HIV negative cohort which had the lowest virus titre. There was no significant association between the presence of dual TTV and TTMV infection and Anellovirus titre in either the bone marrow or spleen tissues.

It has not yet been ascertained if the increased titre of virus in immunosuppressed individuals observed here and in other publications is the result of an increase in viral replication or a decrease of immune mediated viral clearance. A high percentage (71%) of mixed infections with up to 5 isolates of TTV has been noted in HIV infected patients compared with blood donors (21%) and HBV carriers (29%) [Devalle and Niel, 2004]. It is possible that people with compromised immune systems are unable to mount an immune response on exposure to new genotypes of TTV. This would support the finding that stimulation of the immune system with interferon can result in clearance or reduction of TTV viraemia [Akahane et al., 1999; Hagiwara et al., 1999; Maggi et al., 2001]. Published work on HIV-TTV/TTMV co-infections analysed viral loads in serum. Here the analysis has been extended to tissues previously shown to be susceptible to TTV/TTMV.

In conclusion, the data presented here have demonstrated that both TTV and TTMV are present in bone marrow and spleen and the titres of these viruses increases in response to the immunosuppression and decreased CD4 T cell count associated with the progression to AIDS, implying the replication of these viruses is regulated by the immune system. The mechanisms that result in this increased viraemia warrant further investigation however it seems unlikely that the depletion of lymphocytes alone can account for this phenomenon.

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Characteristic	AIDS	Pre-AIDS	HIV negative
Number	13	6	7
Male gender: n (%)	12 (92.3)	3 (50)	4 (57.1)
Age: median (range)	33 (20-60)	29 (25-31)	70 (49-84)
HCV RNA +ve: n (%)	4 (30.7)	2 (33.3)	0
$CD4^+$ T cells/mm^3: median (range)	3 (0-170)	230 (200-247)	NK*

Table I – Characteristics of 3 sample groups used to investigate the prevalence and titre of TTV and TTMV defined by cause of death. * Data not available.

Number of copies of TTV/TTMV per million cells in bone marrow	AIDS	HIV positive, pre-AIDS	HIV negative
$>1x10^8$	3 (23.1%)	0	0
$>1x10^7$	6 (46.1%)	1 (16.6%)	0
$>1x10^6$	4 (30.8%)	1 (16.6%)	0
$>1x10^5$	0	1 (16.6%)	0
$>1x10^4$	0	1 (16.6%)	4 (57.1%)
$>1x10^3$	0	1 (16.6%)	2 (28.6%)
$>1x10^2$	0	1 (16.6%)	0
$>1x10^1$	0	0	1 (14.3%)

Table II – Number of copies of TTV/TTMV per million bone marrow cells as estimated by limiting dilution PCR using primers situated in the UTR region of the TTV and TTMV genome.

Number of copies of TTV/TTMV per million cells in spleen	AIDS	HIV positive, pre-AIDS	HIV negative
$>1x10^8$	1 (7.7%)	0	0
$>1x10^7$	7 (53,8%)	1 (16.6%)	0
$>1x10^6$	1 (7.7%)	1 (16.6%)	0
$>1x10^5$	3 (23.1%)	0	0
$>1x10^4$	1 (7.7%)	2 (33.3%)	3 (42.8%)
$>1x10^3$	0	1 (16.6%)	3 (42.8%)
$>1x10^2$	0	1 (16.6%)	1 (14.3%)
$>1x10^1$	0	0	0

Table III - Number of copies of TTV/TTMV per million spleen cells as estimated by limiting dilution PCR using primers situated in the UTR region of the TTV and TTMV genome.

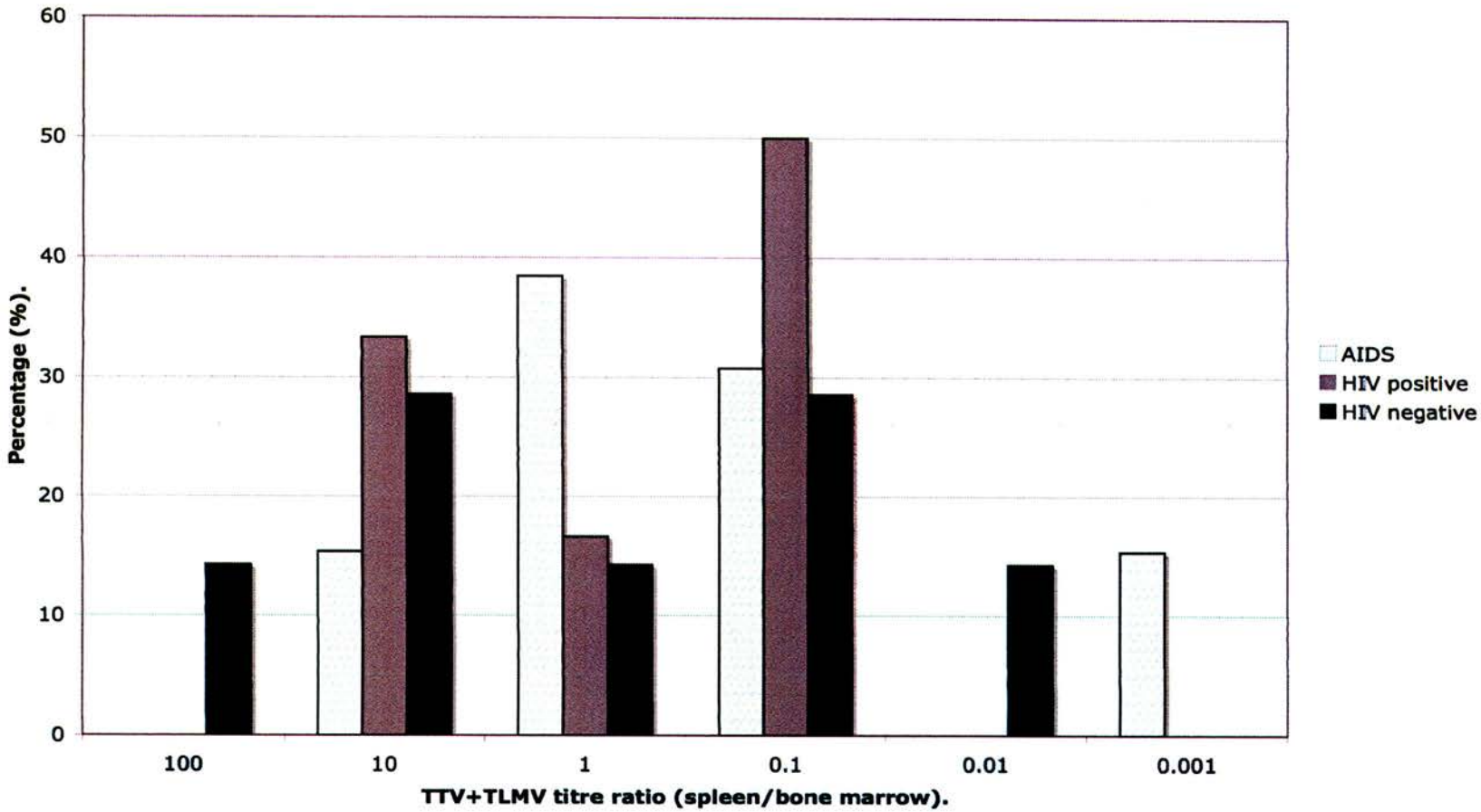

Figure I – Titres of TTV/TTMV in bone marrow and spleen from the 3 groups studied as determined by limiting dilution PCR.

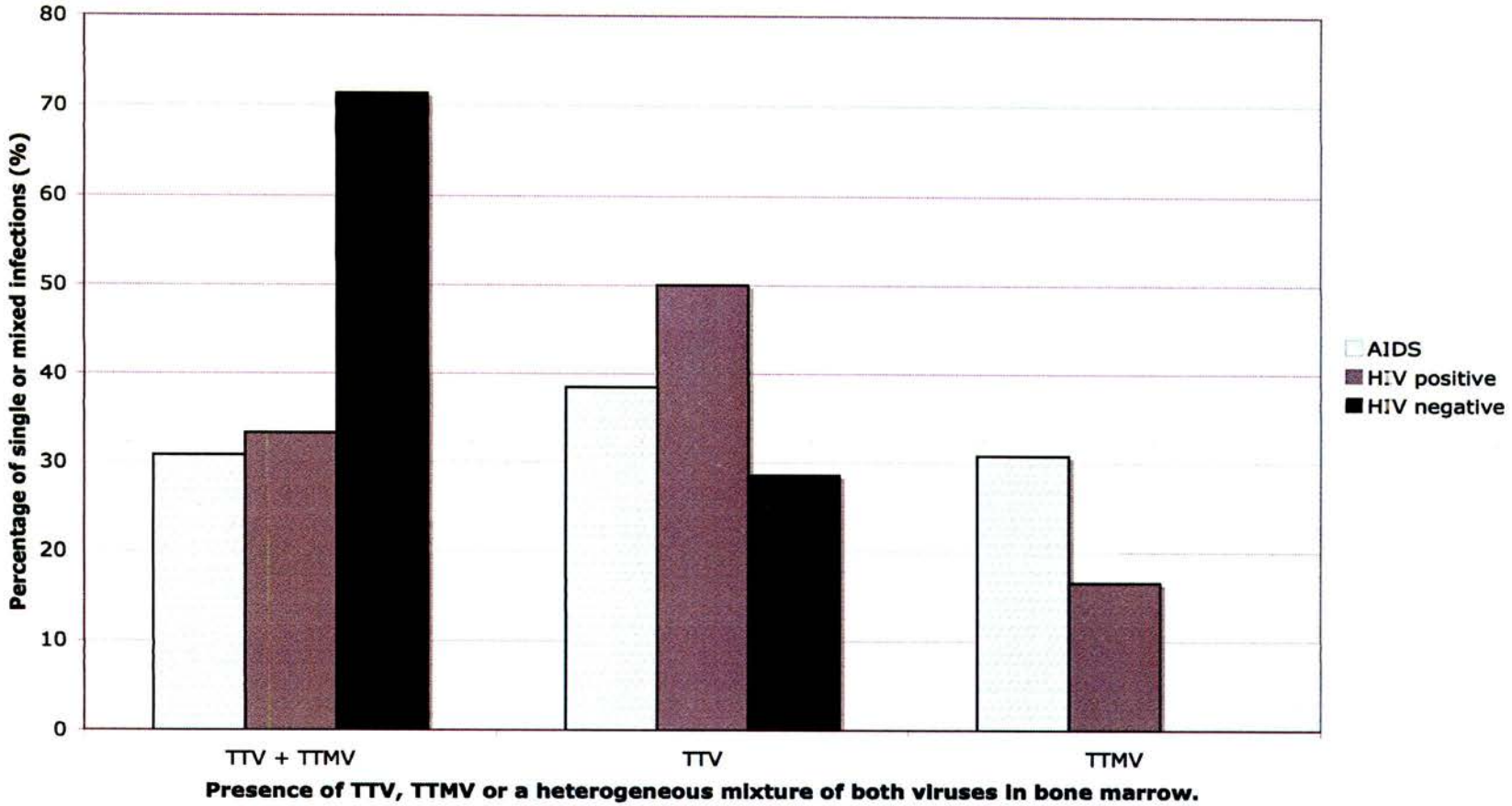

Figure II – Percentage of single TTV and TTMV virus infections and mixed infections in the bone marrow of the AIDS, HIV positive and HIV negative cohorts as determined by amplification and melting curve analysis on a Roche lightcycler.

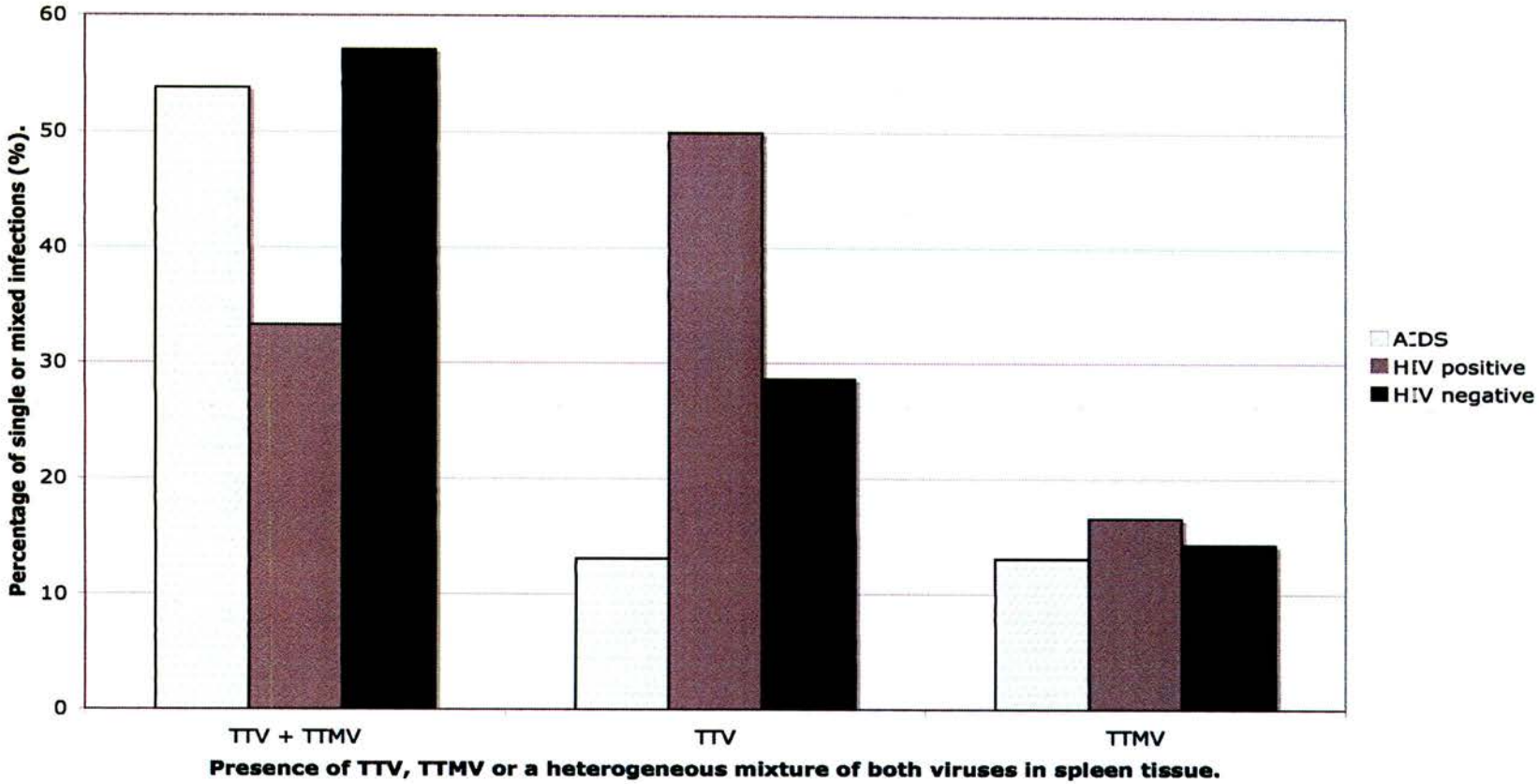

Figure III- Percentage of single TTV and TTMV virus infections and mixed infections in the spleen of the AIDS, HIV positive and HIV negative cohorts as determined by amplification and melting curve analysis on a Roche lightcycler.

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